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Endocannabinoid system and placental development: importance in trophoblast cellular turnover and modulation of protein expression

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na especialidade de Bioquímica

Trabalho realizado sob a orientação de

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Outubro, 2014

Este trabalho foi realizado no Departamento de Ciências Biológica, Laboratório de Bioquímica da Universidade do Porto e no Grupo de Inflamação e Biologia de Reprodução do Instituto de Biologia Molecular e Celular (IBMC) e com o apoio financeiro da Fundação para a Ciência e Tecnologia (FCT), através da atribuição de uma bolsa de doutoramento (SFRH/BD/70721/2010), no âmbito do POPH- QREN- Tipologia 4.1- Formação Avançada, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MEC.

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Publications

Articles in international peer-reviewed journals

M.A. Costa, B.M. Fonseca, N.A. Teixeira, G. Correia-da-Silva. The endocannabinoid anandamide induces apoptosis in cytotrophoblast cells: involvement of both mitochondrial and death receptor pathways. Placenta. 2014. [Epub ahead of print]. DOI: 10.1016/j.placenta.2014.10.011.

M.A. Costa, B.M. Fonseca, E. Keating, N.A. Teixeira, G. Correia-da-Silva. Transient receptor potential vanilloid 1 is expressed in human cytotrophoblasts: induction of cell apoptosis and impairment of syncytialization. The International Journal of Biochemistry & Cell Biology. 2014.[Epub ahead of print]. DOI: 10.1016/j.biocel.2014.10.008.

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Costa MA, Fonseca B, Keating E, Teixeira N, Correia-da-Silva G. 2-arachidonoylglycerol in cytotrophoblasts: Metabolic enzymes expression and apoptosis in BeWo cells. Reproduction. 2014;147:301-11. doi: 10.1530/REP-13-0563.

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M. A. Costa, B. M. Fonseca, N. A. Teixeira and G. Correia-da-Silva. 2-arachidonoyglycerol (2-AG) and placental development: role of endocannabinoids in cytotrophoblast cells turnover (2012). FEBS Journal 279 (Suppl. 1); pp63; P01-37.

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Costa, Mariana A., Keating E., Fonseca B. M., Teixeira N. A., Correia-da-Silva G. The Endocannabinoid System in cytotrophoblast differentiation: 2-arachidonoylglycerol impairs in vitro syncytialization of human cytotrophoblast cells. XLIV Reunião Anual Sociedade Portuguesa de Farmacologia. 5th-8th February 2014. Coimbra (Portugal). (Oral communication).

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M.A. Costa, B.M. Fonseca, V. Guimarães, N. A. Teixeira and G. Correia-da-Silva. The endocannabinoids anandamide and 2-arachidonoylglycerol in placenta: impact in viability and proliferation of trophoblast cells. V SPB Clinical Biochemistry Workshop- Translational Molecular Biochemistry, Coimbra (Portugal), 27 Janeiro, 2012.

Agradecimentos

Às Professoras Doutoras Georgina Correia da Silva e Natércia Teixeira, minhas orientadoras, agradeço os conhecimentos transmitidos, a disponibilidade, o incentivo, o rigor científico e, sobretudo, a oportunidade de realizar este trabalho. À Professora Georgina pela paciência, optimismo e constante motivação que me proporcionou desde o núcleo de investigação da FFUP, quando eu era ainda aluna de Mestrado Integrado, aumentando e cimentando assim o meu gosto pela ciência.

Aos docentes e restantes elementos do Laboratório de Bioquímica da FFUP, nomeadamente ao Bruno, pela paciência e pelas discussões científicas.

À Ana Paula, D. Casimira e ao Daniel pela disponibilidade e eficiência.

À Professora Doutora Elisa Keating, pela sua simpatia e disponibilidade para me transmitir os seus conhecimentos sobre culturas primárias. A todos os estudantes, docentes e funcionários do Serviço de Bioquímica da FMUP, por me terem acolhido e integrado.

Ao Departamento de Obstetrícia e Ginecologia do Hospital de S. João e à Dra. Alexandrina Mendes do Centro Materno-Infantil do Norte pela colaboração neste trabalho.

À Fundação para a Ciência e Tecnologia (FCT), pela bolsa de doutoramento que me foi atribuída (SFRH/BD/70721/2010).

Por último, à minha família, especialmente aos meus pais, pelo incentivo e apoio incondicional.

Abstract

The development of human placenta is a highly dynamic and tightly regulated process that involves proliferation, differentiation and apoptosis of specialized epithelial cells, the trophoblasts. Cytotrophoblasts (CTs) are able to proliferate and to differentiate into the non-proliferative multinucleated syncytiotrophoblast (ST) or develop an invasive phenotype, forming the extravillous trophoblasts, which remodel the uterine tissues and blood vessels. The syncytiotrophoblast is in direct contact with the maternal blood and is responsible for the mother-foetus gas and nutrient exchange and for the placental endocrine function. Anomalies during trophoblast proliferation, differentiation and apoptosis are related to pregnancy-associated complications, such as preeclampsia, intrauterine growth restriction or spontaneous miscarriages.

In the last years, the relevance of the endocannabinoid system (ECS) in different physiological and pathological processes has been recognized. It is constituted by the cannabinoid receptors (CB1 and CB2), their endogenous ligands (endocannabinoids-eCBs) and the biosynthetic and degradation enzymes of the endocannabinoids. The two major endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). They participate in neuroprotection, apoptosis, nociception and reproductive events. In fact, the endocannabinoid signalling is critical for fertility, implantation and decidualization. However, its importance during placental development remains unclear, though CB receptors and AEA metabolic enzymes have already been identified in this organ. In this way, one of the major aims of this work was to search for the presence of other key members of ECS, namely 2-AG metabolic enzymes (DAGL-α, MAGL) and the transient receptor potential vanniloid 1 (TRPV1), an AEA target, in primary human cytotrophoblasts syncytiotrophoblasts and in the cytotrophoblast cell model BeWo. It was demonstrated that these proteins are expressed in these cells. Then, the role of endocannabinoids in cytotrophoblast proliferation, differentiation and apoptosis and the underlying signalling pathways were investigated. AEA and 2-AG induced apoptosis and oxidative stress in BeWo cells, through a CB receptor-dependent mechanism. TRPV1 activation by AEA or its agonist capsaicin (CPS) also triggered apoptosis in human CTs. In addition, 2-AG impaired the biochemical and morphological differentiation of CTs into STs by the activation of CB receptors and these effects may be related to the 2-AG-induced decrease in cyclic AMP (cAMP) levels and protein kinase A (PKA) PKA phosphorylation. CPS also inhibited CT biochemical differentiation through a TRPV1-dependent mechanism, showing the importance of this receptor in cytotrophoblast differentiation. No significant AEA

effects were observed in this process. Collectively, these results contribute to the understanding of the eCBs function during cytotrophoblast cells turnover, namely in apoptosis and differentiation. In addition, the reported evidences suggest that these molecules are new regulators of these cellular events, pointing to a role for ECS and TRPV1 in placental development, and indicate that an abnormal endocannabinoid/ endovanniloid signalling may participate in mechanisms responsible for gestational diseases, such as preeclampsia or spontaneous abortion.

Keywords: apoptosis; cytotrophoblast; endocannabinoid system; placenta; syncytialization; TRPV1

Resumo

O desenvolvimento da placenta humana é um processo altamente dinâmico e extremamente regulado que envolve a proliferação, diferenciação e apoptose de células epiteliais especializadas, os trofoblastos. Os citotrofoblastos são capazes de proliferar e se diferenciar no sinciciotrofoblasto, camada multinucleada desprovida de capacidade proliferativa, ou de desenvolver um fenótipo invasivo, originando os trophoblastos extravilositários, que remodelam os tecidos uterinos e os vasos sanguíneos. O sincíciotrofoblasto está em contacto directo com o sangue materno e é responsável pelas trocas materno-fetais de gases e nutrientes e pela função endócrina placentária. Alterações na proliferação, diferenciação e apoptose dos trofoblastos estão relacionadas com complicações associadas à gravidez, tais como a pré-eclâmpsia, o atraso de crescimento intrauterino ou o aborto espontâneo.

Nos últimos anos, a relevância do sistema endocanabinóide (ECS) tem sido reconhecida em diferentes processos fisiológicos e patológicos. O sistema é constituído pelos receptores canabinóides (CB1 e CB2), pelos seus ligandos endógenos (endocanabinóides-eCBs) e pelas enzimas de biossíntese e de degradação dos endocanabinóides. Os dois principais eCBs são a anandamida (AEA) e o 2-araquidonoilglicerol (2-AG). Estas moléculas participam na neuroproteção, apoptose, nocicepção e em eventos reprodutivos. De facto, a sinalização endocanabinóide é crítica para a fertilidade, implantação e decidualização. No entanto, a sua importância durante o desenvolvimento placentário está ainda por elucidar, embora os receptores canabinóides e as enzimas metabólicas da AEA já tenham sido identificados neste órgão. Deste modo, um dos principais objetivos deste trabalho foi pesquisar a presença de outros membros-chave do ECS, nomeadamente, as enzimas metabólicas do 2-AG (DAGL-α, MAGL) e o receptor de potencial transitório vanilóide do tipo 1 (TRPV1), um alvo da AEA, em culturas primárias de citotrofoblastos (CTs), sinciciotrofoblastos (STs) e no modelo celular de citotrofoblastos BeWo. Foi concluído que estas proteínas são expressas nestas células. Posteriormente, o papel dos endocanabinóides na proliferação, diferenciação e apoptose dos citotrofoblastos e as vias de sinalização subjacentes foram investigados. A AEA e o 2-AG induzem apoptose e stress oxidativo/nitrativo nas células BeWo, por um mecanismo dependente dos receptores canabinóides. A activação do TRPV1 pela AEA ou pelo seu agonista capsaicina (CPS) também induziu apoptose nos CTs. Além disso, o 2-AG inibe a diferenciação morfológica e bioquímica dos CTs em STs, através da activação dos receptores canabinóides e é sugerido que estes efeitos podem estar

relacionados com a diminuição dos níveis de AMP cíclico e da fosforilação da proteína cinase A (PKA) induzida pelo 2-AG. A CPS também inibiu a diferenciação bioquímica dos CTs, por um mecanismo dependente do TRPV1, demonstrando a importância deste receptor na diferenciação dos citotrofoblastos. A AEA não induziu efeitos significativos neste processo. De um modo geral, estes resultados contribuem para a compreensão da função dos eCBs durante a remodelação dos citotrofoblastos, nomeadamente na apoptose e na diferenciação. Além disso, as evidências reportadas sugerem que estas moléculas são novos reguladores destes eventos celulares, sugerindo um papel para o ECS e o TRPV1 no desenvolvimento placentário e indicando que uma sinalização endocanabinóide/endovanilóide alterada pode estar envolvida nos mecanismos responsáveis por doenças relacionadas com a gravidez, tais como a pré-eclampsia ou o aborto espontâneo.

Palavras-passe: apoptose; citotrofoblastos; sistema endocanabinóide; placenta; sincicialização; TRPV1

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∆ψm	mitochondrial membrane potential
12-,15-HETE-EA	12-,15-hydroxyeicosatetraenoic acid ethanolamide
12-,15-HETE-GE	12-,15-hydroxyeicosatetraenoic acid glycerol ester
12-,15-HPETE-EA	12-,15-hydroperoxyeicosatetraenoic acid ethanolamide
12-,15-HPETE-GE	12-,15-hydroperoxyeicosatetraenoic acid glycerol ester
2-AG	2-arachidonoylglycerol
2-AGE	2-arachidonoyl glyceryl ether
3-β-HSD	3-β-hydroxysteroid dehydrogenase
AA	arachidonic acid
ABHD12	α/β-hydrolase 12
ABHD4	α/β-hydrolase 4
ABHD6	α/β-hydrolase 6
AC	adenylyl cyclase
AEA	*N*-arachidonoylethanolamine
AIBP	anandamide intracellular binding proteins
ATF-4	activating transcription factor 4
ATP	adenosine triphosphate
cAMP	cyclic AMP
CB	cannabinoid
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CBD	cannabidiol
c-FLIP	cellular FLICE-like inhibitory protein (c-FLIP)
COX-2	cyclooxigenase 2
CPS	capsaicin
CRE	cAMP response element
CREB	cAMP response element binding protein
CSF	colony-stimulating factor
CT	cytotrophoblast
CYP450	cytochrome P450
DAG	1,2-diacylglycerol
DAGL	diacylglycerol lipase
eCB	endocannabinoid
ECS	endocannabinoid system
EGF	epidermal growth factor
EMT	endocannabinoid membrane transporter

enEVT	endovascular trophoblast
Epac	exchange protein directly activated by cAMP
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESC	endometrial stromal cells
EVT	extravillous trophoblast
FAAH	fatty acid amide hydrolase
FABP	fatty acid binding protein
FAK	focal adhesion kinase
FAN	factor associated with neutral sphingomyelinase activation
FLAT	FAAH-like anandamide transporter
FSH	follicle stimulating hormone
GASP1	G-protein receptor-associated sorting protein
GC	giant trophoblast cell
GCM-1	glial cells missing 1
GDE1	glycerophosphodiesterase 1
GM-CSF	granulocyte-macrophage colony-stimulating factor
GnRH	gonadotrophin-releasing hormone
GPCR	G-protein-coupled receptor
GP-NAE	glycerophospho-*N*-acylethanolamine
GPR55	G-protein-coupled receptor 55
hCG	human chorionic gonadotropin
HERVs	human endogenous retroviral genes
HLA	human leucocyte antigen
hPL	human placental lactogen
HSP	heat shock protein
ICIS	intracytoplasmic sperm injection
ICM	inner cell mass
iEVT	interstitial trophoblast
IGF-1	insulin growth factor 1
IP3	inositol triphosphate
IUGR	intrauterine growth restriction
IVF	*in vitro* fertilization
JNK	c-jun *N*-terminal kinase
LH	luteinizing hormone
LIF	leukemia inhibitory factor
LOX	lipoxygenase
LPA	lysophosphatidic acid
MAG	monoacylglycerol

MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MFSD2a	major facilitator superfamily domain-containing protein 2a
MMP	matrix metalloproteinase
NAAA	*N*-acylethanolamine-hydrolyzing acid amidase
NADA	*N*-arachidonoyldopamine
NAEs	*N*-acylethanolamines
NAGly	*N*-arachidonoylglycine
NAPE	*N*-arachidonoyl-phosphatidylethanolamine
NAPE-PLD	*N*-acylphosphatidylethanolamine-specific phospholipase D
NAT	*N*-acyltransferase
PA	phosphatidic acid
pALP	placental alkaline phosphatase
PARP-1	poly (ADP-ribose) polymerase 1
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG-EA	prostaglandin-ethanolamide
PG-GE	prostaglandin-glyceryl ester
PI	phosphatidylinositol
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PLA1	phospholipase A1
PLA2	phospholipase A2
PLC	phospholipase C
PP13	placental protein 13
PPAR	peroxisome proliferator-activated receptor
PTPN22	protein tyrosine phosphatase non-receptor type 22
PTX	pertussis toxin
RNS	reactive species of nitrogen
ROS	reactive species of oxygen
RXR	retinoid X receptor
SHIP1	SH2 domain-containing inositolphosphatase
SM	sphingomyelin
SMase	sphingomyelinase
ST	syncytiotrophoblast
STAT3	signal transducer and activator of transcription 3
TASK-1	TWIK-related acid-sensitive K^+ channels 1
tBid	truncated Bid

TGF	transforming growth factor
THC	Δ^9-tetrahydrocannabinol
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRIB-3	telomere repeat binding factor 3
TRPV1	transient receptor potential vanilloid 1
uPA	urokinase plasminogen activator
VGCC	voltage-gated calcium channels
VSMC	vascular smooth muscle cells
XIAP	X-linked inhibitor of apoptosis

PART I

Introduction

1. Development and functions of human placenta

A new human life begins with the fertilisation of an oocyte by a spermatozoa, giving rise to the first cell of the new being, the zygote. Then, the zygote goes through successive mitosis originating the morula, which undergoes compaction forming the blastocyst. The blastocyst has an outer epithelial cell layer, the trophectoderm, which will form the placenta and extra-embryonic tissues; and an inner cell mass (ICM), which will give rise to the embryo. The blastocyst implantation occurs after 5-7 days of fertilization. For a successful implantation, it is required that the endometrium becomes receptive to the blastocyst, after differentiation of endometrial stromal cells (ESCs) into decidual cells. In humans, the decidualization occurs every menstrual cycle, in the late secretory phase, to prepare the endometrium for the implantation. If fertilisation does not occur, decidua is expelled during the menstruation and the endometrium is regenerated. If fertilisation occurs, the decidua is maintained and the endometrium undergoes further changes that include vascular and glandular remodelling and the appearance of the uterine immune cell populations.

The formation of placenta begins with the differentiation of the trophectoderm in different trophoblasts, the specialized epithelial cells of placenta. In the *prelacunar stage*, trophoblast cells proliferate and differentiate into the multinucleated syncytiotrophoblast (ST), which has an invasive phenotype in this stage, allowing the blastocyst penetration into the decidua. The remaining mononuclear cells are the cytotrophoblasts (CT) and act as stem cells, permitting the growing of ST layer. In the *lacunar stage*, fluid-filled spaces appear in the syncytiotrophoblast and merge, forming the lacunae. The lacunae are surrounded by the trabeculae (a ST mass), which will be important for the villous trees formation. At this stage, CTs invade the trabeculae and reach the maternal tissues, giving rise to the extravillous trophoblasts (EVTs). In the *villous stage*, the trabeculae begin to branch and contain a core filled with CT, forming the primary villi. Then, the extraembryonic mesodermal cells go along with the CTs and invade the trabeculae, though not reaching the maternal side, giving rise to the secondary villi. The mesodermal cells differentiate into haematopoietic cells and, then, blood vessels independent on embryo vascular system emerge, originating the tertiary villi [1].

At microscopic level, placenta is then constituted by villous trees that keep emerging from the continuous branching of trabeculae. Some of them contact with the basal plate (region facing the endometrium) and contain the trophoblastic cell columns (anchoring villi), while other end freely in the intervillous space (floating villi) [1]. This placental

microarquitecture allows an intimate contact between mother and foetus, since the maternal blood is in direct contact with trophoblast foetal cells, due to the deep invasion of maternal tissues. This type of placentation is called haemochorial [2].

Macroscopically, the human term placenta is a discoid organ with about 22 cm of diameter but there is a considerable interindividual variability of its anatomy. The basal plate is divided in lobes/cotyledons, which contain the chorionic villi that arise from the foetal side of placenta (the chorionic plate) and are irrigated by foetal blood vessels [1].

The placental development is a dynamic and highly regulated process and anomalies in this gestational event will impair the important functions of human placenta, such as gas and nutrient exchanges and embryo protection [3], compromising the success of pregnancy outcome. The main functions of this organ are summarized in Figure 1.

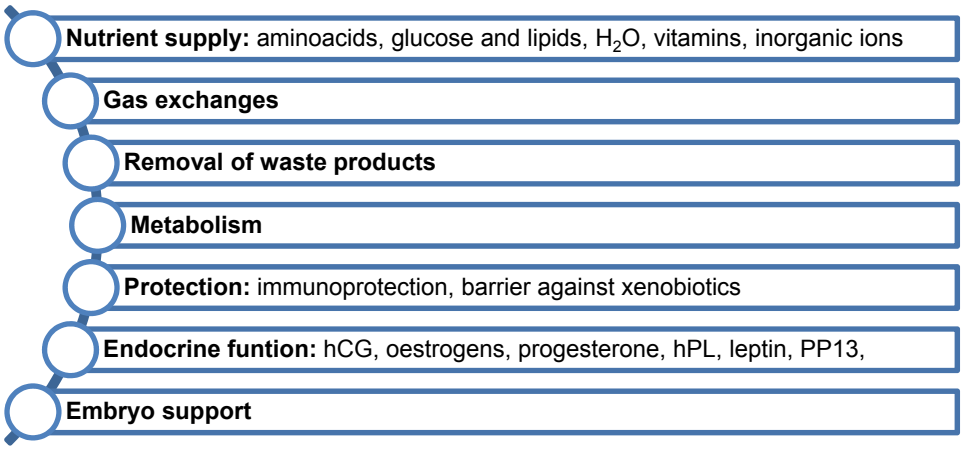


Figure 1. Main functions of human placenta.
hCG- human chorionic gonadotropin; hPL- human placental lactogen; PP13- placental protein 13.

1.1 The trophoblast

In placental environment, there are several cell types that contribute to placental structure and function, including trophoblasts, decidual cells, mesenchymal cells, Hofbauer cells (placental macrophages) and endothelial cells [1]. The trophoblasts are the main placental cells. In human placenta, there are four types of trophoblasts: cytotrophoblast, syncytiotrophoblast, extravillous trophoblasts, and giant trophoblast cells. They have different morphologies, properties and functions, which turns the placenta into a unique organ. Figure 2 represents the mother-foetal interface, with the structure of human chorionic villi and the trophoblast cell types.

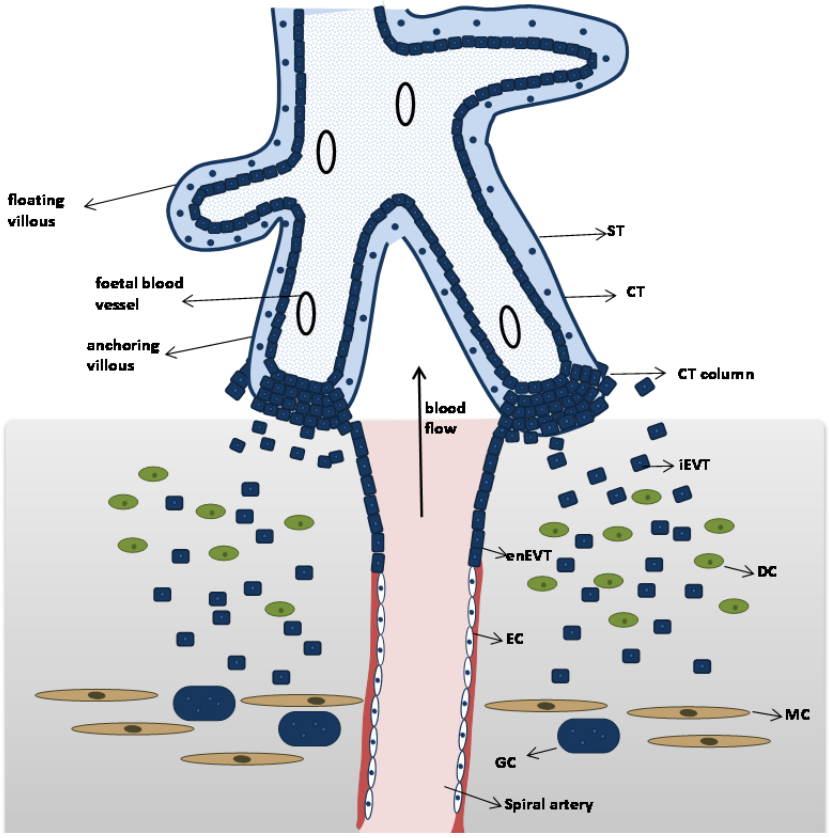


Figure 2. Representation of the mother-foetal interface.
CTs are able to proliferate and fuse to originate a multinucleated layer, the ST. In the anchoring villi, CT form a cytotrophoblast cell column. Then, CTs differentiate into invasive trophoblasts, which will remodel the maternal decidua (iEVTs) and blood vessels (enEVTs), enlarging the spiral arteries caliber and consequently diminishing the resistance to the blood flow that irrigates the foetus. In the myometrium, to limit their invasion into maternal tissues, iEVTs fuse, giving rise to the multinucleated GCs. CT- cytotrophoblast; DC-decidual cell; EC- endothelial cell; enEVT- endovascular extravillous trophoblast; GC- giant trophoblast cell; iEVT- interstitial extravillous trophoblast; MC- myometrial cell; ST-syncytiotrophoblast.

The *villous cytotrophoblasts* are the first trophoblast cell type to differentiate from the trophectoderm. They are mononuclear cells that proliferate, differentiate into other trophoblast cell types and die by apoptosis. Their proliferation is essential for the placental development and maintenance. CT proliferation is regulated by oxygen and growth factors and is higher in the first trimester, when the oxygen tension is lower, decreasing in the following trimesters [4].

Cytotrophoblast differentiation occurs by two pathways: the villous and the extravillous pathways (Figure 3). In the villous pathway, CTs proliferate, aggregate, fuse and undergo biochemical differentiation to originate the syncytiotrophoblast; this process is called syncytialization. In the extravillous pathway, CTs proliferate and acquire invasion properties, which allow the invasion of uterine arteries (endovascular trophoblasts; enEVTs) and endometrium (interstitial trophoblasts; iEVTs) [3, 5, 6].

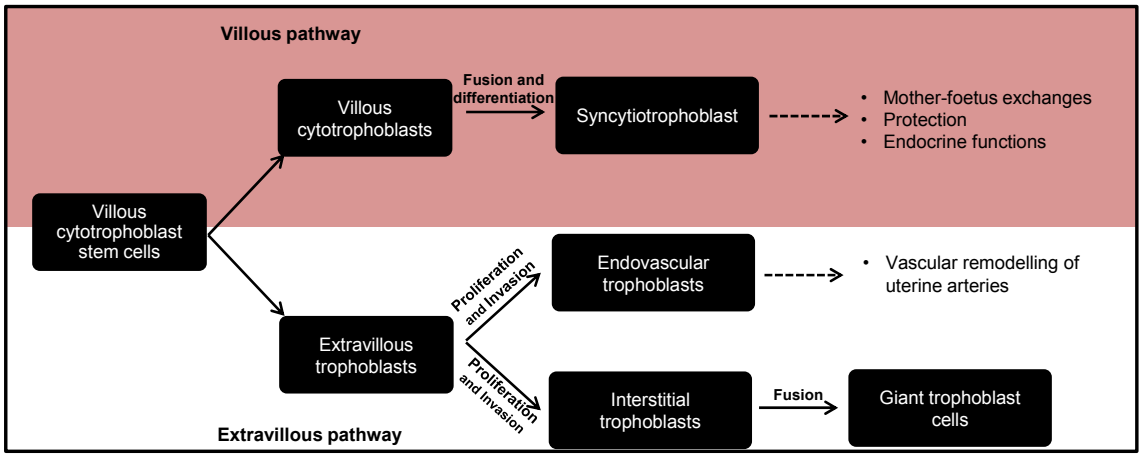


Figure 3. Schematic representation of the differentiation pathways of human cytotrophoblasts.

The *extravillous trophoblasts* are mononucleated cells with invasive capacity that remodel the uterine tissues and maternal spiral arteries. The iEVTs invade the decidua and the inner third of the myometrium. There, they aggregate and fuse, forming the placental bed *giant trophoblast cells* (GCs). These cells are multinucleated cells that are not able to migrate and invade and their formation is considered a regulatory mechanism of trophoblast invasion since prevents a deeper penetration into the uterine wall [7]. iEVTs induce a loss of vascular smooth muscle cells (VSMC) of spiral arteries and enEVTs invade the spiral artery, replace endothelial cells and form plugs in the spiral artery, creating a hypoxic environment in the first trimester. EVTs induce caspase-dependent apoptosis in VSMC via TNF-related apoptosis-inducing ligand (TRAIL) and in endothelial cells via TRAIL and FasL. These changes aim to increase the blood vessel calibre and, concomitantly, diminish the resistance to the blood flow, facilitating the gas and nutrient exchange [8]. The spiral arteries remodelling is a key event during placental development since an insufficient invasion is associated with gestational diseases like preeclampsia [9].

The trophoblast invasion involves restructuration of extracellular matrix and so, EVTs show a distinct profile of adhesion molecules (e. g. increased expression of some integrins or VE-cadherin and lower expression of E-cadherin). Furthermore, they express proteases like matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) that are able to degrade the extracellular matrix. On the other hand, they also express protease inhibitors, tissue inhibitors of metalloproteinase (TIMPs) and plasminogen activator inhibitors, which regulate EVTs invasion into maternal tissues. Several growth factors and other proteins have a stimulatory (e. g. leptin, activin A, epidermal growth factor EGF) or inhibitory (transforming growth factor TGF-β, inhibin A)

effect on EVTs invasion. Besides trophoblasts, other placental cells like decidual cells, uterine NK cells and activated macrophages also produce some of these factors, participating in the control of EVTs invasion. Moreover, EVTs express a distinct human leucocyte antigens (HLA) profile, which contributes to the immunotolerance at the maternal-foetus interface [9].

Besides enEVTs and iEVTs, recent evidences indicate that there is another EVT population, the endoglandular trophoblasts, suggesting that uterine glands undergo remodelling, which aims the opening of glands lumen to the intervillous space, providing histiotrophic nutrition to the embryo before the uteroplacental circulation during the early placental development [10].

1.1.1 Syncytialization

The cytotrophoblast differentiation into syncytiotrophoblasts is an event that begins a few days after implantation and continues till the end of pregnancy. The mononuclear CTs undergo morphological and biochemical differentiation to form a non-proliferative multinucleated cell layer that directly contacts with the maternal blood, the syncytiotrophoblast, which is the only barrier that separates mother and foetal blood. This syncytial layer is maintained by continuous fusion of the underlying cytotrophoblast cells and is involved in the foetal nourishment, protection and protein biosynthesis.

The mononucleated CTs aggregate and fuse into the ST multinucleated layer. In this process, there is a rearrangement of the cytoskeleton with α-fodrin fragmentation and actin reorganization and decrease of E-cadherin expression [11, 12]. These changes are enhanced by the activation of the transcription factor glial cells missing 1 (GCM-1) [13, 14]. GCM-1 regulates the transcription of the human endogenous retroviral genes (HERVs), *HERV-W* and *HERV-FRD*, which codify the fusogenic proteins syncytin-1 and 2, respectively [13, 15-18]. The biochemical or functional differentiation is responsible for the ST expression of proteins involved in transport, metabolism and production of several hormones and enzymes, which are essential for a successful pregnancy outcome, such as human chorionic gonadotropin (hCG), human placental lactogen (hPL), leptin, aromatase and placental alkaline phosphatase (pALP) [6, 19].

Cytotrophoblast fusion and differentiation is regulated by several signalling pathways. Some of the factors that modulate the syncytialization are summarized in Table 1.

Table 1. Factors that modulate the syncytialization.

Factor	Specification	Effect in syncytialization	Ref.
Connexin-43	gap-junction protein	↑ Bd/Md*	[20, 21]
EGF	growth factor	↑ Bd/Md	[22, 23]
CSF	growth factor	↑ Bd/Md	[24]
GM-CSF	growth factor	↑ Bd/Md	[24]
IGF-1	growth factor	↑ Bd	[25]
TNF-α	cytokine	↓ Bd/Md	[26]
TGF-α	growth factor	↑ Bd/Md	[27]
TGF-β	growth factor	↓ Bd	[28, 29]
Oestradiol	hormone	↑ Bd/Md	[30]
hCG	hormone	↑ Bd/Md	[31, 32]
Adiponectin	adipocytokine	↑ Bd/Md	[33]
Activin	growth factor	↑ Bd	[29]
LIF	cytokine	↑or↓ Bd; ↑Md*	[34-37]
Caspase-8	protease	?	[38, 39]
Caspase-14	protease	↓Bd/Md*	[40, 41]
GCM-1	transcription factor	↑ Bd/Md	[13, 14]
Syncytin-1	endogenous retroviral envelope protein	↑ Bd/Md	[15, 18]
Syncytin-2	endogenous retroviral envelope protein	↑ Bd/Md	[16]
Cadherin-11	cell adhesion protein	↑ Bd*/Md	[42]
PPAR-γ	nuclear receptor	↑ Bd/Md	[43, 44]
RXR-α	nuclear receptor	↑ Bd	[45, 46]
CD98	membrane protein; amio acid transporter	↑ Md*	[47, 48]
MFSD2a	syncytin-2 receptor	↑ Md*	[49]
cAMP	second messenger	↑ Bd/Md	[50, 51]
p38	kinase	↑ Bd/Md	[52]
p42/p44	kinase	↑ Bd/Md	[52]
PKA	kinase	↑ Bd/Md	[50, 51]
Src family kinases	kinases	↑ or↓Bd/Md	[53]
Epac	guanine nucleotide exchange factor	↑ Bd/Md	[54, 55]
Calponin 3	cytoplasmic protein	↓ Md*	[56]
Galectin-1	lectin	↑ Md*	[57]
Galectin-3	lectin	↑ Md*	[47]
RhoE	Rho GTPase family protein	↑ Md*	[58]
Intermediate conductance Ca2+-activated K^+ channels	potassium ion channels	↓ Bd/Md	[59]
voltage-gated K^+ channels	potassium ion channels	↓ Bd	[60]
Stathmin	microtubule regulatory protein	↑ Bd/Md*	[61]
Syndecan-1	membrane-bound proteoglycan	↑ Bd/Md*	[62]
Twist	transcription factor	↑ Md*	[63]
ZO-1 (zona ocludens 1)	membrane proteins	↑ Bd/Md	[64]
Hypoxia	environment condition	↓ Bd/Md	[65, 66]

↑-stimulation, ↓-inhibition; ?- inconsistent; Bd-biochemical differentiation; Md- morphological differentiation; *data obtained with cytotrophoblast cell lines.

The signalling cascade cyclic AMP/ protein kinase A/cAMP response element binding protein (cAMP/PKA/CREB) is one of the major interveners in the ST formation. In fact, during this process, cAMP levels increase leading to phosphorylation of PKA and, consequently, of CREB, promoting CTs biochemical differentiation [50, 67-71]. In addition, cAMP has been related to CTs morphological differentiation, since it regulates the expression of GCM-1 and syncytin-1 and 2 [51, 72]. Besides the activation of PKA, increased cAMP levels may also induce CT fusion and differentiation by the activation of the exchange protein directly activated by cAMP (Epac) signalling pathway [54, 55, 73].

The phosphorylation of mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-regulated kinase 1/2 (ERK 1/2) also triggers the cytotrophoblast differentiation [52]. In fact, p38 phosphorylation mediates EGF stimulation of syncytialization [23], whereas phosphorylation of both p38 and ERK 1/2 are involved in the biochemical differentiation induced by the inhibition of Src family kinase [74]. Moreover, p38 regulates the expression of peroxisome proliferator-activated receptor (PPAR-γ) [75], whose activation also triggers both fusion and biochemical differentiation of CTs, by stimulating the secretion of hCG, hPL and leptin and the transcription of GCM-1 and syncytin-1 [43, 44, 46, 76].

Some authors defend that the exposure of phosphatidylserine to the outer leaflet of plasma membrane and caspase 8 activation, are involved in CTs plasma membrane remodelling, contributing to their fusion [38, 77]. However, other evidences raised questions about the role of caspase-8 in this process and suggest that the activation of this protease is not required for syncytialization [39, 78].

1.1.2 Apoptosis in trophoblast

Besides proliferative and differentiation events, the apoptotic cell death of trophoblasts is another important cellular process for placental development, since it removes the quiescent cells without inducing an inflammatory reaction, allowing the proliferation and differentiation of new cells. However, the mechanisms involved in the regulation of trophoblast apoptosis still need to be clarified. It was reported that an abnormal expression of apoptotic proteins, such as Bax, Bcl-2 or FasL are associated with pathological conditions like preeclampsia, HELLP syndrome, IUGR and hyperglycaemia [79-84]. There are some controversies about the trophoblast phenotypes that undergo apoptosis. Huppertz *et al*. proposed that the CTs only present early stage apoptotic features, such as caspase-8 activation and phosphatidylserine exposure in the outer

leaflet of plasma membrane, features that lead to cell fusion. Moreover, they express high levels of the anti-apoptotic protein Bcl-2, which inhibits the progression of the apoptotic cascade. Later, after fusion into the ST, this cascade is reactivated and the aged and apoptotic syncytiotrophoblast nuclei are aggregated into protrusions of the apical membrane, forming the syncytial knots. Then, these structures are extruded and released into the maternal blood [85]. However, other authors reported that CTs are the main cells that undergo apoptosis and the apoptotic markers that apparently were localized in the ST were actually from apoptotic CTs, which were highly interdigitated into the ST [86]. Also, recent evidences suggest that apoptosis inhibition in ST is important because, once initiated, it would widespread throughout the entire syncytial layer, leading to placental dysfunction [87]. The EVTs also undergo apoptosis, though few information is known about this process. Nevertheless, it seems that decidual, endothelial and local immune cells may modulate EVTs apoptosis [85].

The proteins required to trigger the mitochondrial and death receptor pathways are expressed in CTs, STs and EVTs [88, 89]. In normal conditions, cytotrophoblasts do not undergo apoptosis after Fas activation [90, 91], probably because these cells express high levels of X-linked inhibitor of apoptosis (XIAPs) and cellular FLICE-like inhibitory protein (c-FLIP) [88]. On the other hand, CT cells induce a Fas and TRAIL-mediated apoptosis in T cells and VSMC, respectively [92, 93].

1.2 Placental hormones

The placental hormones are important throughout pregnancy, since they play a role in pregnancy establishment and maintenance, foetal development and labour. The major source of placental hormones is the syncytiotrophoblast layer. The ST has the enzymatic machinery required for the biosynthesis of several hormones, which are not expressed in other trophoblast phenotypes.

1.2.1 Human chorionic gonadotropin (hCG)

The human chorionic gonadotropin (hCG) is one of the most important pregnancy-related hormones. It is a glycoprotein that shares similarities with the hypophysary luteinizing hormone (LH). Besides the hCG hormone characteristic of pregnancy, there are other 4 variants of this protein, with similar amino acid sequences but produced by

different cells and with different functions: sulphate hCG, hyperglycosylated hCG, hCG free β-subunit and hyperglycosylated hCG free β-subunit.

The pregnancy-produced hCG act on LH/hCG receptors and its main functions include stimulation of progesterone secretion by corpus luteum, angiogenesis of uterine vasculature, promotion of the CTs fusion and differentiation into ST, immunosuppression and prevention of myometrial contractions. hCG is detectable from day 8 after ovulation and its levels peak at 10th week of pregnancy, declining slowly till the end of the gestational period [94]. As it is almost exclusively produced during pregnancy (there are some cancerous cells that produce this hormone), the detection of this hormone in blood and urine is the most common biomarker used in pregnancy tests.

The signalling pathways involved in hCG biosynthesis are already elucidated. The cAMP/PKA pathway stimulates hCG production [50, 71]. On the other hand, the activation of LH/hCG receptor also enhances adenylyl cyclase (AC) activity, increasing cAMP levels [95]. Moreover, Src kinases are also able to modulate hCG secretion [53] and, in the cytotrophoblast cell model BeWo cells, a crosstalk between cAMP/PKA and MAPKs p38 and ERK1/2 pathways mediates the forskolin-induced increase in hCG levels, promoting CT differentiation [96].

1.2.2 Progesterone

Progesterone is a steroid essential for pregnancy maintenance. It is synthesised from cholesterol through a two-step reaction occurring in trophoblast mitochondria: cholesterol side-chain cleavage by cytochrome P450 (CYP450scc) converts maternal cholesterol into pregnenolone, which is metabolized to progesterone by 3-β-hydroxysteroid dehydrogenase (3-β-HSD) [97]. At the beginning of gestation, luteal corpus is the main producer of progesterone but from 6th week of gestation till the end of pregnancy, placenta becomes the main source of this steroid and progesterone serum levels increase till the end of gestation. Progesterone acts in several events throughout gestational period: stimulates decidualization and myometrial relaxation and quiescence, enhances the expression of profertility Th2 cytokines in decidual cells, blocks the oestrogens pro-proliferative effect in the uterine cells, promotes embryo attachment, modulates hCG synthesis and represses MMPs activity, impairing trophoblast invasion. It acts by binding to two types of receptors: nuclear and membrane-bound progesterone receptors [98, 99].

1.2.3 Oestrogens

Placental oestrogens are steroids that consist in four different hormones: oestrone, oestradiol, oestriol and oestretol. On the contrary to other steroidogenic organs, placenta is not able to convert pregnenolone and progesterone into androgens, since 17α-hydroxylase/17, 20-lyase is absence in this organ. In this way, placenta uses circulating androgens that essentially come from foetal adrenal glands (but also from the mother), and the enzyme CYP450 aromatase expressed in the syncytiotrophoblast converts them into oestrogens [97]. Aromatase activity is modulated by cAMP levels [100]. Oestradiol promotes implantation, vasodilatation and increases uterine contractions in labour. However, oestradiol actions in the trophoblasts remain unclear. Moreover, oestradiol enhances syncytialization, induces leptin expression and may, indirectly, modulate EVTs function [98, 101].

The oestriol is the most abundant oestrogen in the urine. It is considered a weak oestrogen, though it is important for increasing uteroplacental blood flow and is one of the hormones tested for screening foetal anomalies [97].

1.2.4 Leptin and other adipokines

Adipokines are adipocyte-derived signalling proteins that, in the last years, have emerged as important regulators of gestational events, namely in placental development.

Leptin is the most well-known adipokine in pregnancy. Serum levels of leptin are increased in pregnant women, peaking in late 2nd and early 3rd trimester. The main source of leptin is the syncytiotrophoblast and leptin receptors are also expressed in this multinuclear layer. This hormone has pleotropic effects in pregnancy, such as in implantation, angiogenesis, embryo development, stimulation of hCG secretion, CT proliferation and induction of MMP-2 and MMP-9 synthesis and, consequently, EVTs invasion. Leptin triggers JAK/STAT, ERK1/2 and Phosphoinositide 3-kinase (PI3K) pathways [102]. Its expression is regulated by other pregnancy hormones like hCG, hPL, insulin, progesterone, oestrogens and by the second messenger cAMP. In fact, cAMP induces the expression of leptin, in a mechanism that also involves a crosstalk between PKA and ERK1/2 pathways [103]. Additionally, hCG enhances the production of leptin through a mechanism that involves an interplay between cAMP and p38 [104] and also by the Epac/cAMP pathway [105].

Adiponectin is another adipokine whose importance in placentation has been recognized. It exerts several tissue-specific effects, partially depending on the several

isoforms found in the circulation: trimmers, hexamers and high molecular weight multimers. Its serum levels are increased in early pregnancy and then decline in the second trimester [106]. Adiponectin diminishes the endocrine function of term syncytiotrophoblast, since it decreases hCG, progesterone and hPL secretion [107]. On the other hand, it enhances syncytialization in first trimester [33] and has antiproliferative effects on trophoblastic cell lines, BeWo and JEG-3 [108]. Moreover, adiponectin stimulates the migration and invasion of first trimester EVTs, through the upregulation of MMP-2 and -9 and downregulation of TIMP-2 [109].This cytokine also inhibits insulin signalling and insulin-stimulated amino acid transport [110] through the activation of PPAR-α and ceramide synthesis [111].

Resistin serum levels are increased in the 3rd trimester of gestation. This adipokine is expressed in EVTs and increases MMP-2 whereas decreases TIMP-1 and -2, promoting cell invasion. Resistin also stimulates angiogenesis [106].

2. The Endocannabinoid System

The consume of *Cannabis sativa* either for medicinal or recreational purposes began a thousand years ago in central Asia. The medicinal properties of this plant were first explored in China, India, Egypt, Syria, Persia and Tibet, where it was used for the treatment of malaria, constipation, rheumatism, pain, absentmindedness, menstrual fatigue, sleep disorders [112-114]. However, the first cannabinoids were only identified in the 60's: the Cannabidiol (CBD) [115] and the most psychoactive constituent of cannabis, Δ^9- tetrahydrocannabinol (THC) [116].

After the identification of THC, an intense research about its mechanism of action began. The strong hydrophobicity of this molecule suggested that its actions resulted from non-specific interactions with cell membrane [117]. However, the synthesis of THC enantiomers and its synthetic analogues, as well as the discovery that THC was enantioselective indicated that THC effects were receptor-dependent. In 1988, the first cannabinoid receptor (CB1) was identified in rat brain by Devane *et. al.* [118] and in 1992, the first endogenous ligand of this receptor, the *N*-arachidonoylethanolamine or Anandamide (AEA), was discovered in porcine brain [119]. Then, in 1993, the second cannabinoid receptor CB2 was identified in human promyelocytic leukemia cells (HL-60 cells) [120]. These discoveries and the identification of other endogenous ligands for CB1 and CB2 contributed to unveil the existence of the Endocannabinoid System (ECS). Nowadays, the members that constitute this system are the cannabinoid receptors (CB1 and CB2), their endogenous ligands (endocannabinoids – eCBs), the enzymes involved in synthesis and degradation of these ligands and the eCBs putative membranar transporter (EMT). In Figure 4, the members of ECS are represented, as well as their role in the system.

In the last years, the ECS has been studied in several scientific fields and it has emerged as an important intervener in different physiological and pathophysiological mechanisms. Actually, drugs targeting the members of ECS have been explored as therapeutic alternatives for the treatment of pathological conditions such as pain, cancer, obesity, inflammation and neurodegenerative disorders [121]. The drugs that target ECS are divided in several families such as direct agonists or antagonists of CB receptors and inhibitors of eCBs biosynthesis, degradation or reuptake [122]. Cannabinoid agonists, like THC or the synthetic cannabinoid nabilone are legally used in some countries to treat nausea and emesis in cancer patients undergoing chemotherapy and to induce appetites in AIDS patients. These compounds have also proved efficacy in the management of

interferon and ribavirin-induced anorexia, nausea and weight loss in chronic hepatitis C patients. In Portugal, a spray containing THC and cannabidiol (CBD), Sativex®, is commercialized since 2012 for the treatment of spasticity in multiple sclerosis patients [123]. The selective antagonist of CB1 receptor, Rimonabant (Acomplia®) was a promisor anti-obesity drug and was commercialized in several countries. However, Acomplia® was discontinued due to its psychiatric side effects (anxiety and depression) [124].

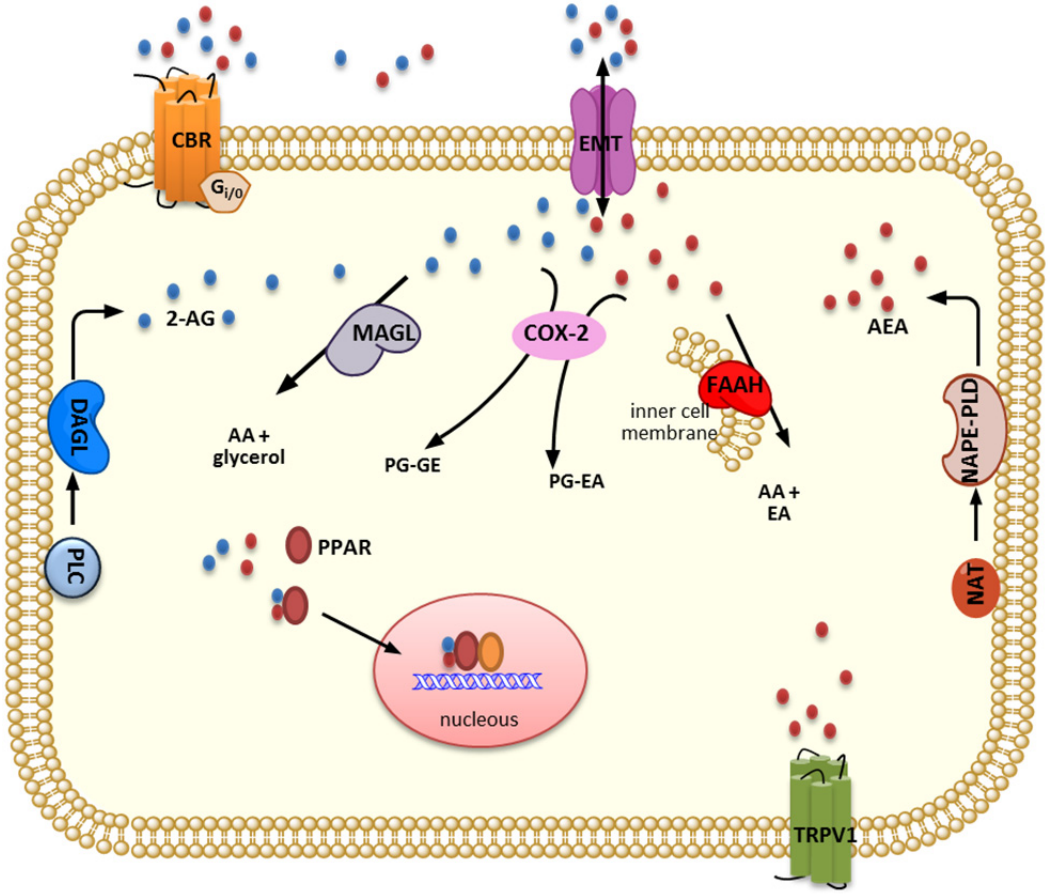


Figure 4. Biochemistry of the endocannabinoid system (ECS).
Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthetized from membrane precursors. AEA is synthesised by the enzymes *N*-acyltransacylase (NAT) and *N*-acylphosphatidoylethanolamine- Phospholipase D (NAPE-PLD), whereas the synthesis of 2-AG is catalysed by Phospholipase C (PLC) and Diacylglicerol lipase (DAGL). Both eCBs are released to the extracellular environment by diffusion or selective endocannabinoid membrane transporter (EMT), where they activate G.protein-coupled cannabinoid receptors (CBR). Then, they are rapidly internalized also by diffusion /EMT and are degraded. AEA is mainly hydrolysed by fatty acid amid hydrolase (FAAH), releasing ethanolamine (EA) and arachidonic acid (AA) or is oxidized by Cyclooxygenase 2 (COX-2), to prostaglandin-ethanolamides (PG-EA). 2-AG is converted to glycerol and AA mainly by Monoacylglycerol lipase (MAGL) or is oxidized by COX-2 to prostaglandin-glyceryl esters (PG-GE). In addition, AEA is capable of activating the cation channel transient receptor potential vanilloid 1 (TRPV1) receptor in the intracellular side and both AEA and 2-AG are able to bind to the nuclear receptors peroxisome proliferator-activated receptor (PPAR), modulating the transcription of their target genes.

2.1 Endocannabinoids

By definition, endocannabinoids are endogenous lipid molecules that are capable of binding to and functionally activate cannabinoid receptors, exerting similar cellular effects to the most psychoactive constituent of *Cannabis sativa*, the THC.

As abovementioned, the first eCB *N*-arachidonoylethanolamide (Anandamide, AEA) was identified in 1992 in porcine brain [119]. In the following years, other eCBs have also been identified: 2-arachidonoylglycerol (2-AG) [125, 126], 2-arachidonoyl glyceryl ether (2-AGE) [127], *O*-arachidonoylethanolamine (virodhamine) [128], *N*-arachidonoyldopamine (NADA) [129], *N*-arachidonoylglycine (NAGly) [130] and Oleamide [131]. Chemically, these molecules are amides, esters or ethers of long-chain polyunsaturated fatty acids and are structurally different from THC but share critical pharmacophores [132, 133]. They are synthesised in cytoplasm through multiple biosynthetic pathways and released into the extracellular environment, where they bind and activate CB receptors. After exerting their action, eCBs are internalized and degraded [134]. The molecular structure of THC and eCBs are represented in Figure 5.

All eCBs have different affinities for cannabinoid receptors and exhibit differences in their pharmacological profile comparing with THC and synthetic cannabinoids. Some eCBs are able to activate other receptors like the Transient Receptor Potential Vanilloid Type-1 (TRPV1) and PPAR-α and γ [135].

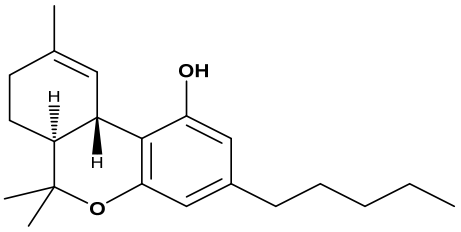
Anandamide and 2-AG are the best characterized members of the main eCBs families, the *N*-acylethanolamides (NAE) and monoacylglycerols (MAG), respectively. Since the pharmacology and metabolism of these compounds are the most well-known, they are considered as ‘major’ endocannabinoids.

Besides eCBs, there are other endogenous molecules, “endocannabinoid-like”, which are able to modulate the endocannabinoid signalling, although they are devoid of activity on CB receptors. These molecules are denominated eCB-like compounds and share the hydrolysing enzymes with the eCBs, exacerbating their effects by an ‘entourage’ effect, due to the competitive inhibition of these enzymes [136].

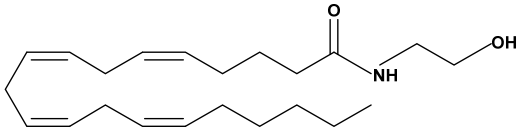
2.1.1 Anandamide (AEA)

N-arachidonoylethanolamine or Anandamide is the best-characterized eCB. The name Anandamide comes from the Sanskrit word *ananda*, which means ‘internal bliss’ [137]. As the other NAEs, this lipid is an endogenous eicosanoid derivative, which is synthesized from phospholipids of cell membrane and arachidonic acid, linked by an amide bound. Its

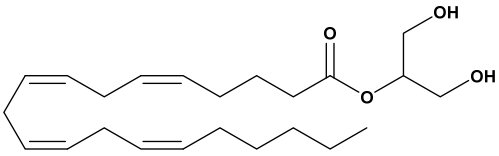
main biosynthetic enzyme is *N*-acyl-phosphatidylethanolamine (NAPE-PLD), whereas the main hydrolysing enzyme is Fatty Acid Amide Hydrolase (FAAH) [134].



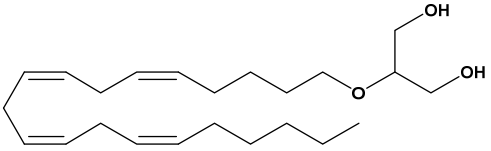
Δ^9-Tetrahydrocanabinol (THC)



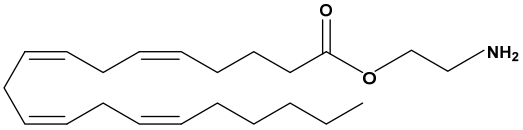
Anandamide (AEA)



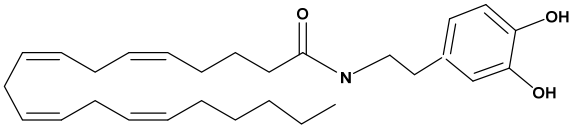
2-Arachidonoylglicerol (2-AG)



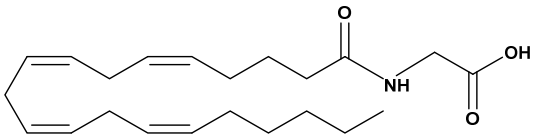
2-Arachidonoylglicerol Ether (2-AGE)



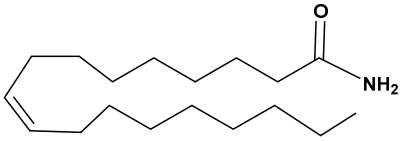
Virodhamine



N-arachidonoyldopamine (NADA)



N-arachidonoylglycine (NAGly)



Oleamide (ODA)

Figure 5. Chemical structures of the psychoactive phytocannabinoid THC and of the endogenous cannabinoids.

It is known that AEA is very important in numerous physiologic processes like modulation of different stages of reproduction [138-141], nociception [142, 143], muscle relaxation [144, 145], inflammation [146, 147], stimulation of appetite [148, 149], cell differentiation [150-152] and apoptosis [138, 153-155]. For a long time, researchers thought that AEA and other eCBs were synthetized 'on demand'. However, recent data

demonstrated that AEA can be stored inside the cell in adiposomes [156]. Moreover, AEA can interact with soluble carrier proteins facilitating its trafficking [157, 158].

Anandamide acts as partial agonist of both cannabinoid receptors, which is unusual for an endogenous ligand. Like THC, AEA has lower efficacy and affinity for CB2 than for CB1 [159]. Besides cannabinoid receptors, AEA can also activate TRPV1, a non-selective cation channel [160] and so, this eCB is also considered an endovanilloid. This receptor can also be responsible for some AEA cellular effects, like vasodilation and apoptosis [161]. AEA can also activate the nuclear receptors PPAR-α [162] and PPAR-γ [163]. Interestingly, AEA is able to downregulate the levels of the other major eCB, 2-AG, through activation of TRPV1 in postsynaptic neurons, resulting in an inhibition of 2-AG synthesis by Diacylglycerol Lipase α (DAGL-α) [164].

In this way, AEA is capable of acting inside the cell through the activation of TRPV1 and PPARs and exerting an autocrine and/or paracrine effect on CB receptors when it is released from the cell [165]. AEA in the extracellular fluid can reach different targets with the help of protein carriers like serum albumin and lipid-binding proteins, such as lipocalins [166], acting as an endocrine messenger [165].

2.1.2 2-arachidonoylglycerol (2-AG)

2-arachidonoylglycerol was an already known endogenous metabolite when, in 1995, Mechoulam *et al*. identified it in canine gut and described its intrinsic activity on CB1 and CB2 [125]. Sugiura *et al*. also found that this eCB binds to the cannabinoid receptors in rat brain synaptosomes [126]. There are emergent evidences that 2-AG is an important messenger in multiple physiological processes such as neuroprotection [167, 168], inflammation [169-171], nociception [172, 173], reproductive events [174, 175], cell differentiation [176, 177] and apoptosis [175, 178]. Similarly to AEA, 2-AG is synthesised in the cytoplasm through different pathways and the most relevant is the one involving the enzyme diacylglycerol lipase (DAGL). The major 2-AG hydrolysing enzyme is monoacylglycerol lipase (MAGL) [134].

2-AG binds to CB1 and CB2 receptors with higher efficacy than AEA but the affinity for CB2 is slightly lower than for CB1 [159]. Unlike AEA, 2-AG acts as full agonist in various systems and seems that its structure is strictly recognized by the cannabinoid receptors, which leads some authors to conclude that 2-AG is the true natural ligand for the CB receptors [179]. It has been referred that the concentration of 2-AG in the brain is between 170 [180] to 800 [126] times higher than AEA. Also, in mouse uterus and in rat decidual

cells, 2-AG levels are, respectively, 200- and 150-fold higher than those of AEA [174, 175]. In contrast with AEA, 2-AG seemed to be a very weak activator of TRPV1 [160], though recent evidences support that this eCB is also a ligand of this receptor [181, 182]. 2-arachidonoylglycerol, as AEA, activates PPAR-γ [163].

2.2 Receptors for the endocannabinoids

Cannabinoid receptors, CB1 and CB2, are members of the superfamily of G protein-coupled receptors (GPCR), which have a seven-domain transmembrane structure. Their ligands bind to the extracellular domain, activating different cellular pathways. Initially, it was thought that THC effects resulted from its interaction with cellular membranes, which would stimulate or inhibit membrane-associated enzymes and alter the physical state of ion channels [117, 183]. However, the finding that THC induced an inhibition of AC and a decrease in the levels of cAMP in neuroblastoma cells constituted the first evidence of the existence of cannabinoid receptors [184, 185]. These inhibitory effects were blocked by pertussis toxin (PTX), suggesting the involvement of $G_{i/0}$ proteins [186]. In 1988, a study using a radiolabelled and enantiomerically pure analogue of THC allowed the identification of specific binding sites in the brain, the CB1 receptors [118]. In 1990, these receptors were cloned from rat brain [187]. Later, in 1993, the discovery of CB2 demonstrated that it only shares 44% of amino acid sequence homology with CB1 [120]. While CB1 receptor is highly conserved across the human, rat and mouse species, CB2 protein structure is divergent, which may have implications for future pharmacological studies involving CB2 [188]. Initially, it was thought that CB1 was only localized in brain and that CB2 was restricted to immune system, however, their distribution is more ubiquitous. Besides brain, CB1 is peripherally expressed in several organs, including spleen, tonsils, bladder, small intestine, sympathetic nerve terminals, smooth muscle cells and reproductive tissues [134]. CB2 receptor is mainly expressed in periphery especially in lymphoid organs and immune cells but it was also identified in brain tissues [189].

Other difference between the two cannabinoid receptors is the physical association of CB1 with lipid rafts in several cell types, since these membrane microdomains are important for the modulation of its activity [190-193]. In fact, it was reported that the disruption of lipid rafts with methyl-β-cyclodextrin enhances the AEA-induced effects through CB1 by interfering with mechanisms responsible for attenuation or termination of CB1 signalling [192]. On the other hand, cholesterol enrichment of plasma membrane reduced the CB1 receptor-dependent signal transduction, due to the increased rigidity

[194]. Also, lipid rafts are important for CB1 internalization by endocytosis [190, 195, 196], which leads to degradation in lysosomes or recycling, regulating CB1 levels in the cell membrane. On the contrary to CB1, CB2 binding and signalling is not dependent on the microarchitecture of the plasma membrane [197-199]. This may result from the putative cholesterol recognition amino acid sequence present in CB1 [200] or due to the post-translational palmitoylation of this receptor [201], that seem to be important for CB1 interaction with lipid rafts. Nevertheless, it was also described that lipid rafts disruption reverses AEA-induced cell death in different cell types, through receptor-independent mechanisms [138, 202, 203].

In the last years, evidences supporting the existence of other putative cannabinoid receptors have been emerged. The orphan GPCR, GPR55, is the best candidate to be considered the third cannabinoid receptor (CB3), since it seems to be activated by cannabinoid receptors agonists, including AEA and 2-AG [204].

Besides cannabinoid receptors, it has been reported that endocannabinoids interact with other type of receptors and modulate the activity of ion channels. Transient Receptor Potential Vanilloid (TRPV1) is a non-selective cation channel belonging to the six-transmembrane-domain Transient Receptor Potential (TRP) channels and it was identified in 1997 by Caterina *et al.* [205]. This receptor is activated by noxious heat (> 42 ºC), low pH (<6) and by several endogenous and exogenous molecules and its best known agonist is the capsaicin (CPS), an hot chilli pepper component [206]. TRPV1 is expressed in several organs and tissues among the body and participates in different physiological events. In fact, this receptor is mainly involved in temperature sensing [207] and nociception [205, 208, 209], but is also associated with other cellular processes such as apoptosis [153, 210, 211], muscle contraction [212-214], cell differentiation [215-217], autophagy [218, 219] and inflammation [208, 220, 221].

It is known that AEA and 2-AG may also target the nuclear receptors PPARs involved in the regulation of metabolism and energy homeostasis, cell differentiation, immune/inflammatory responses and reproductive events. [162, 163, 222-224].

2.3 Metabolism of Endocannabinoids

The endocannabinoids, mainly AEA and 2-AG, are involved in several important physiological mechanisms, so their biosynthesis and degradation must be tightly regulated to maintain the homeostasis of endocannabinoid signalling.

2.3.1 Biosynthesis of Anandamide and other *N*-acylethanolamines

N-acylethanolamines like AEA can be synthetized through multiple pathways (Figure 6). They are originated from plasma membrane phospholipids [225] and their major biosynthetic pathway, transacylation–phosphodiesterase pathway, is catalysed by the enzymes *N*-acyltransacylase (NAT) and *N*-acylphosphatidoylethanolamine-Phospholipase D (NAPE-PLD). NAT is a calcium-dependent enzyme localized in cellular membranes, which transfers a fatty acid chain from sn-1 position of 1, 2-diacylglycerophospholipid (e.g. phosphatidylcholine –PC) to the amine group of phosphatidylethanolamine (PE). From this reaction results the precursor of NAE, *N*-acylphosphatidylethanolamine (NAPE), and a lysophospholipid [226]. NAPEs are biological constituents of plasma membrane and their levels are increased in inflammation and tissue degeneration [226].

The second step of NAE formation is catalysed by NAPE-PLD, a calcium-dependent and highly conserved enzyme belonging to the zinc metallo-β-lactamase family. This enzyme is also localized in biological membranes and converts *N*-arachidonoylphosphatidylethanolamine (NArPE) into AEA and other NAPEs into their correspondent NAE, releasing phosphatidic acid [227].

Although the transacylation-phosphodiesterase pathway is considered the major source of AEA, NAPE-PLD knockout mice revealed the existence of alternative biosynthetic pathways [228]. One pathway involves the double *O*-deacylation of NAPEs by the serine hydrolase α/β-hydrolase 4 (ABHD4), which is expressed in several organs. ABHD4 hydrolyses NAPE to lyso-NAPE and then lyso-NAPE to glycerophospho-*N*-acylethanolamine (GP-NAEs) [229]. GP-NAE is finally converted into NAEs by the metal-dependent GP-NAE phosphodiesterase, glycerophosphodiesterase 1 (GDE1) [230]. In addition, secreted Phospholipase A2 (sPLA2) is expressed in several rat tissues especially in stomach and is also able to convert NAPE to lyso-NAPE, which is then converted to AEA by lyso-Phospholipase D (lyso-PLD) [231].

Another pathway of AEA synthesis involves the synthesis of phospho-*N*-arachidonoylethanolamine (pAEA) by a not yet identified Phospholipase C (PLC)–like enzyme. pAEA is subsequently dephosphorylated by a protein tyrosine phosphatase (PTPN22), originating AEA. Inositol 5'-phosphatase SHIP1 also contributes to the synthesis of AEA from pAEA [232].

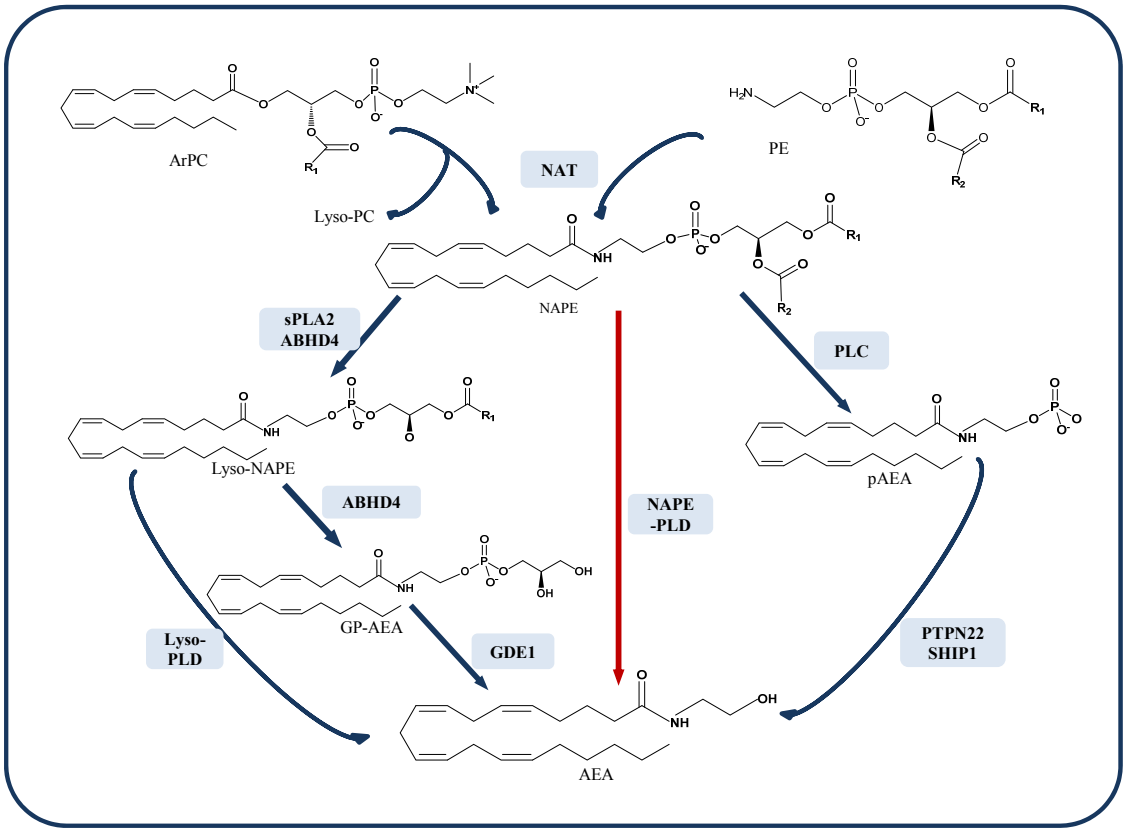


Figure 6. Biosynthetic pathways of Anandamide (AEA).
AEA is produced from membrane phospholipids by NAT, resulting the intermediate NAPE. Then, NAPE is hydrolysed by several enzymes, being NAPE-PLD the major biosynthetic enzyme. ABHD4-α/β-hydrolase 4; ArPC- arachidonoylphosphatidylcholine; GDE1- glycerophosphodiesterase 1; GP-AEA- glycerophospho-AEA; lyso-NAPE- lysophosphatidyl-NAPE; lyso-PC- lysophosphatidylcholine; NAPE-PLD- *N*-acylphosphatidylethanolamine-phospholipase D; NAT- *N*-acyltransferase; pAEA -phospho-AEA; PE-phosphatidylethanolamine; PLC- phospholipase C; PTPN2- phosphatase protein tyrosine phosphatase 22; SHIP1- SH2 domain-containing inositol phosphatase. sPLA2-secreted phospholipase A2.

2.3.2 Biosynthesis of 2-Arachidonoylglycerol

The most significant biosynthetic pathway of 2-AG is a two-step reaction catalysed by PLC and Diacylglycerol Lipase (DAGL), which converts arachidonic acid-containing membrane phospholipids, such as phosphatidylinositol (PI) into 2-AG (Figure 7).

PLC generates 1-acyl-2-arachidonoylglycerol or Diacylglycerol (DAG) and the latter is hydrolysed by DAGL [126, 233]. More recently, two DAGL enzymes (α and β) were cloned and characterized. They are expressed in the membrane fraction, their optimal activity is achieved at pH 7 and are stimulated by Ca^{2+} [179]. Alternatively, phospholipase A1 (PLA1) can synthetize 2-arachidonoyl-lysophosphatidylinositol (lyso-PI), which is hydrolysed to 2-AG by lyso-PLC activity [234]. Under certain conditions, 2-AG can also be synthesized through the conversion of 2-arachidonoyl lysophosphatidic acid (LPA) by a

phosphatase [235]. Finally, the intermediate of 2-AG synthesis, DAG, can alternatively be produced from phosphatidic acid (PA) by PA hydrolase [236].

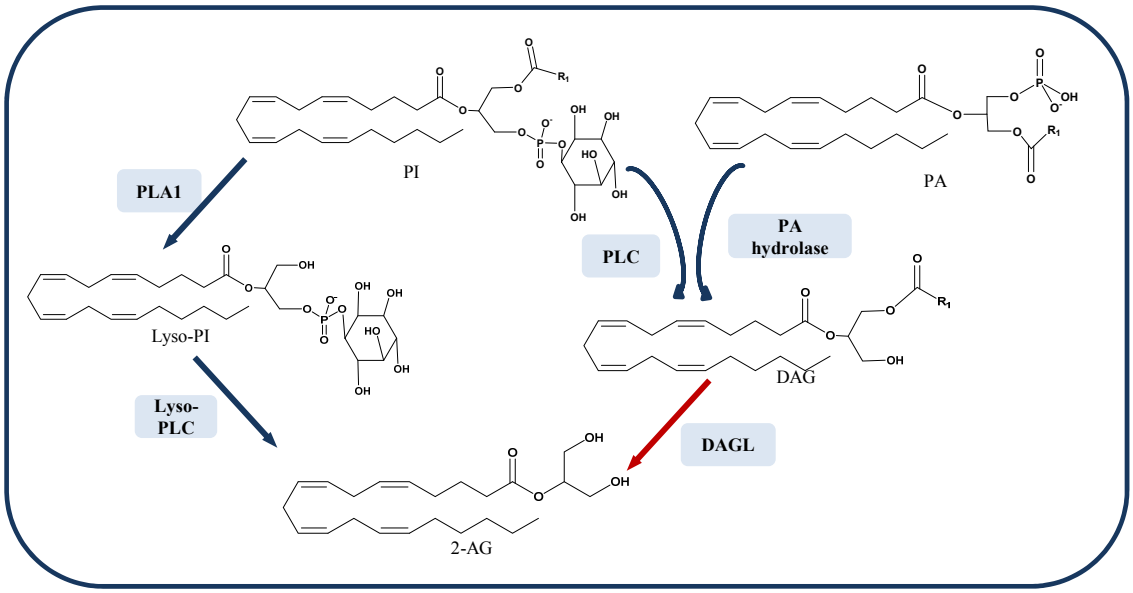


Figure 7. Biosynthetic pathways of 2-arachidonoylglycerol (2-AG).
The main synthetic pathway is a two-step reaction involving the formation of intermediate DAG by PLC, followed by DAGL hydrolysis. Alternatively, PLA1 and Lyso-PLC can also synthetize 2-AG. 2-AG can also be produced from PA by PA hydrolase. DAG-diacylglycerol; DAGL-diacylglycerol lipase; Lyso-PI- lyso phosphatidylinositol; lyso-PLC-lysophosphatidylinositol phospholipase C; PA- phosphatidic acid; PI-phosphatidylinositol; PLA1-phospholipase A1.

2.3.3 Degradation of Anandamide and other *N*-acylethanolamides

The pathways involved in AEA degradation are summarized in Figure 8. FAAH hydrolyses AEA to arachidonic acid (AA) and ethanolamine (EA) and is the most important degrading enzyme [237]. FAAH is a membrane-bound enzyme of the serine hydrolases family, achieves maximal activity at pH 9 and is a member of amidase signature family of enzymes characterized by a Ser241-Ser217-Lys142 catalytic triad [238]. This enzyme has been cloned in several species and has an ubiquitous expression, though it is preferentially expressed in brain, small intestine and testis [239, 240]. FAAH is expressed in intracellular membranes and is mainly found in microsomal and mitochondrial fractions [240]. It hydrolyses amides and esters at same rate, contrasting with other serine hydrolases, which express greater activity for esters [241].

Recently, other isoform of FAAH, FAAH-2, was identified. These two isoforms are both members of amidase protein family, own an *N*-terminal transmembrane domain and share the Ser-Ser-Lys catalytic triad but only have approximately 20% of sequence identity

[239]. FAAH-2 is expressed in mammalian tissues but not in rodent tissues and it is the predominant isoform in heart and ovaries [239]. In contrast with FAAH-1, FAAH-2 is not localized in the endoplasmic reticulum (ER) and is expressed in cytosolic lipid droplets, suggesting that these enzymes may have different physiological roles [242]. In addition, FAAH-2 is much less effective than FAAH-1 at hydrolysing NAEs [239], but its expression in peripheral tissues suggests that this molecule might have a rescue role in NAEs hydrolysis upon FAAH inactivation [238]. Moreover, C-terminal catalytic domains of FAAH-1 and FAAH-2 are in cytoplasmic and luminal compartments of the cells, respectively, suggesting that this opposite orientation might influence their access to specific fatty acid amide substrates in cells, especially if these lipids show preferential localization to the inner or outer leaflet of the membrane bilayer [239].

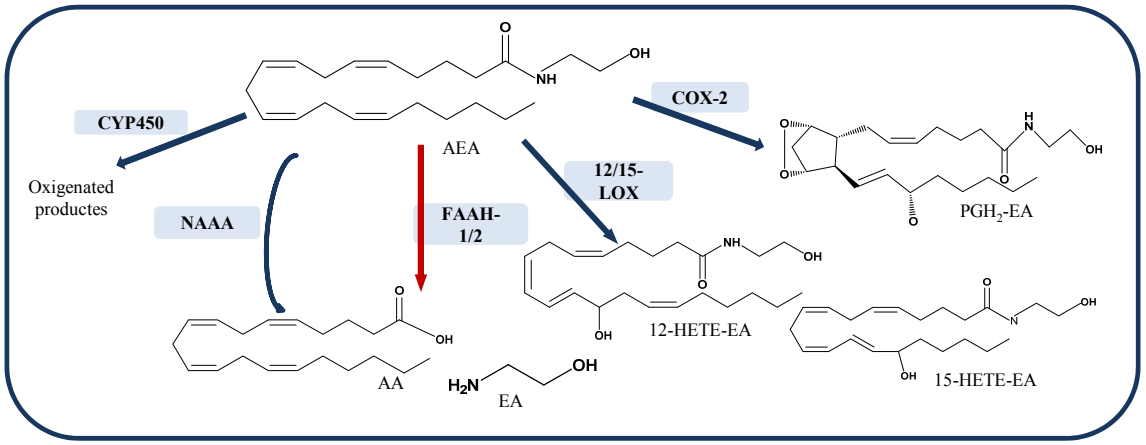


Figure 8. Inactivation pathways of Anandamide (AEA).
AEA can be inactivated by hydrolysis to AA and EA by FAAH-1 (the major pathway), FAAH-2 and NAAA or can be oxidized by COX-2, 12- and 15-LOX or CYP450. AA-arachidonic acid; COX-2-cyclooxigenase 2; CYP450-cytochrome P450; EA-ethanolamine; FAAH 1/2-fatty acid amide hydrolase 1/2; 12-, 15-HETE-EA-12-, 15-hydroxyeicosatetraenoic acid ethanolamide;12-,15-LOX-12-,15-Lypoxigenase; NAAA- *N*-acylethanolamine-hydrolysing acid amidase; PGH_2-EA- prostaglandin-ethanolamides.

N-acylethanolamine-hydrolysing acid amidase (NAAA) is an acid ceramidase-like protein that hydrolyses NAEs. The first evidences of the existence of NAAA emerged with the report of a FAAH-distinct AEA hydrolysis activity in human megakaryoblastic cell line [243]. NAAA is mainly localized in lysosomes, is self-activated by a acid pH-dependent proteolytic cleavage and has a cysteine residue responsible for its catalytic activity [244].

Besides hydrolysis, AEA may also be metabolized by the same enzymes that are responsible for the oxidation of arachidonic acid, including Cyclooxygenase-2 (COX-2), 12- and 15-Lipoxygenase (12-LOX, 15-LOX) and Cytochrome P450 (CYP450). COX-2

oxidizes AEA to several oxygenated products (prostaglandin-ethanolamides (PG-EA) or prostamides) [245]. Although their lack of affinity for CB receptors [246], some of these metabolites are provided with biological activity, mediating AEA-induced apoptosis [247-250] and inflammation [251, 252]. 12- and 15- LOX induce a hydroxylation in the AEA molecule, forming the hydroperoxides 12- and 15-hydroperoxyeicosatetraenoic acid ethanolamide (12-HPETE-EA, 15-HPETE-EA), which will be enzymatically or chemically reduced to the corresponding hydroxyeicosatetraenoic acid, 12-HETE-EA and 15-HETE-EA [253]. LOX metabolites of AEA seem to be agonists of TRPV1 receptor [254] and are powerful natural inhibitors of FAAH, controlling AEA degradation [255]. Also, 12-HETE-EA binds to CB receptors with an affinity similar to that of AEA [256]. CYP450 is also able to oxidize AEA to epoxyeicosatetraenoyl-ethanolamides and one of these compounds is able to activate CB2 receptor [257].

2.3.4 Degradation of 2-Arachidonoylglycerol

The major enzyme responsible for 2-AG degradation is the serine hydrolase Monoacylglycerol Lipase (MAGL). This enzyme cleaves 2-AG into AA and glycerol, owns a Ser122-His269-Asp239 catalytic triad and is ubiquitously expressed [258]. In brain, MAGL is localized in presynaptic neurons so, it has been pointed as the pivotal enzyme controlling the duration of 2-AG-mediated retrograde signalling [259]. MAGL is localized in cytosol and is responsible for 85% of degradation of 2-AG in rat brain [260]. The remaining 15% are mostly catalysed by the enzymes α/β-hydrolase domain 6 and 12 (ABDH6, ABDH12). Interestingly, MAGL, ABHD6, and ABHD12 display distinct subcellular distributions, suggesting that they may control distinct pools of 2-AG in the nervous system [261]. Moreover, 2-AG is also a substrate for FAAH due to the esterase activity of this enzyme [262] and, although FAAH seemed to have a reduced contribution for 2-AG hydrolysis in brain [261], recent evidences pointed that FAAH is relevant for the regulation of 2-AG levels in hepatic cells [263].

Similarly to AEA, 2-AG may also be oxidized by COX-2, 12- and 15-LOX. COX-2 converts 2-AG to Prostaglandin-glyceryl esters (PG-GE), like PGE_2-GE [264], while the oxidation by 12- and 15-LOX produces 12- and 15-hydroperoxyeicosatetraenoic acid glycerol ester (12-HPETE-GE, 15-HPETE-GE), respectively, which will originate the corresponding hydroxyeicosatetraenoic acids, 12-HETE-GE and 15-HETE-GE [253].. Some of these oxygenated metabolites have biological activity [265-268]. 15-HETE-GE seems to have a biological role due to its agonist activity in PPAR-α [269]. LOX

metabolites of 2-AG were found to be powerful natural FAAH inhibitors and might play a role in controlling AEA degradation [255]. CYP450 can also oxidize 2-AG and the produced metabolites play a role in Ca^{2+}-induced relaxation of rat mesenteric arteries [270]. The degradation pathways of 2-AG are resumed in Figure 9.

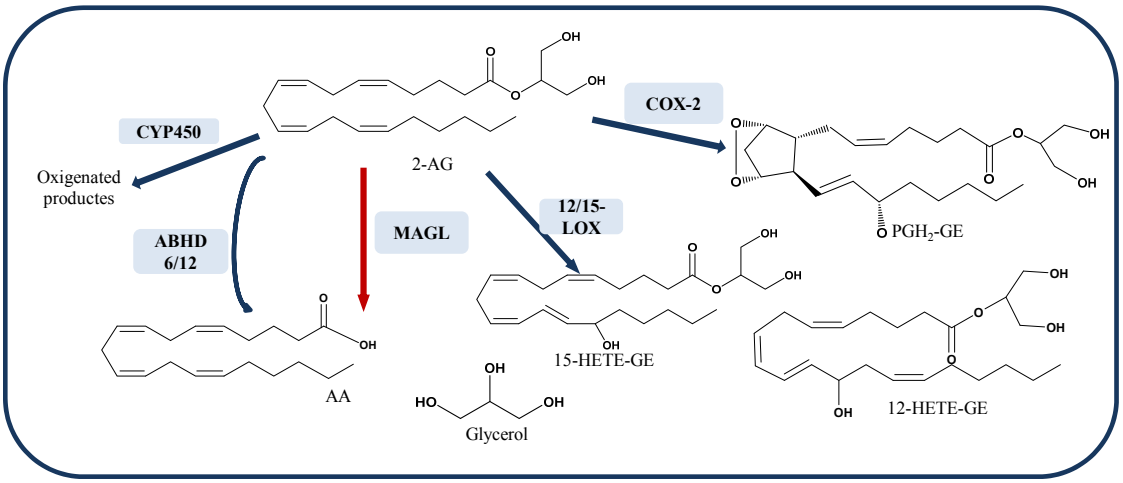


Figure 9. Inactivating pathways of 2-AG.
2-AG is mainly hydrolysed by MAGL to AA and glycerol but ABHD6/12 can also hydrolyse this EC. In addition, 2-AG might also be oxidized by COX2, 12- and 15-LOX and CYP450. AA-arachidonic acid; ABHD6/12-α/β-hydrolase 6/12; CYP450-cytochrome P450; COX-2-cyclooxigenase 2; 12-,15-HETE-GE-12-,15-hydroxyeicosatetraenoic acid glycerol ester; 12-,15-LOX- 12-,15-Lypoxigenase; MAGL-monoacylglycerol lipase; PGH_2-GE- prostaglandin-glycerol esters.

2.3.5 Release and Uptake of Endocannabinoids

After biosynthesis, eCBs are released to extracellular space to activate CB receptors either by membrane diffusion or by a transporter, or they can act inside the cell through TRPV1 or PPARs activation. Cessation of cannabinoid signalling occurs through a two-step process comprising the transport of eCBs from the extracellular to the intracellular space followed by degradation through hydrolysis or oxidation. It has been proposed that eCBs are able to diffuse through cell plasma membrane due to their lipophilic nature but, on the other hand, other data indicate that the uptake of eCBs is a transmembrane protein-facilitated process [271].

For years, it has been proposed that AEA crosses cell membrane by a membrane-localized protein carrier, the EMT. This transporter has been characterized as temperature-dependent, saturable, bidirectional and energy-independent, however, the type of protein involved is not yet identified [272-274]. Recently, Fu *et al*. identified a truncated variant of FAAH-1 in neurons. This protein was designated FAAH-1 like

anandamide transporter (FLAT), lacks amidase activity but binds to AEA, facilitating its uptake [275]. However, other results raised questions about FLAT as an AEA intracellular transporter, since they reported that this protein has residual catalytic activity and is localized in intracellular membranes [276].

The simple diffusion is another proposed mechanism of AEA uptake, since it can interact with membrane phospholipids and the membrane cholesterol seems to facilitate AEA uptake [192, 277]. Therefore, some authors defend that there is no membrane protein carrier and that the transport of AEA across cell membrane is conditioned via its intracellular hydrolyses by FAAH and/or its intracellular sequestration [278]. The continuous degradation by FAAH maintains the concentration gradient necessary to drive the process of simple diffusion [274, 278]. However, other data reported that FAAH is not the only responsible for eCBs uptake, as cells and tissues of FAAH knockout mice are also able to accumulate AEA by a saturable, selective, pharmacologically inhibited and bidirectional mechanism [272].

The intracellular sequestration of eCBs by binding to intracellular proteins has also been suggested as a mechanism for eCBs uptake [279] but only, recently, these proteins were identified. AEA can interact with soluble carrier proteins facilitating the trafficking of AEA. In fact, albumin, Heat Shock protein 70 (HSP 70), Fatty Acid Binding proteins 5 and 7 (FABP5, FABP7) and FLAT are Anandamide Intracellular Binding Proteins (AIBP) and seem to have an important role in intracellular sequestration, rapid and efficient distribution of AEA to the different intracellular compartments [157, 158, 275]. Very recently, it was also found that the sterol carrier protein-2 may participate in AEA's cellular uptake though, due to its low affinity for this eCB, it does not contribute significantly to its intracellular sequestration [280]. Furthermore, AEA can also be accumulated in adiposomes [156] and so its half-life inside the cell may be extended, in order to achieve higher concentrations and last long enough to activate nuclear PPARs. Therefore, it has been proposed that AIBP may act as anandamide intracellular transporters, carrying AEA to different cellular compartments, like adiposomes, where it can be accumulated and/or degraded by FAAH, COX-2 and 15-LOX, enzymes that may also be localized in these cellular structures [165]. This intracellular sequestration supports the simple diffusion of AEA, since the removal of free AEA from the intracellular pool promotes the inward concentration gradient and, consequently, the AEA uptake [279]. On the other hand, recently, it was reported that EMT transport is independent from AIBPs or degrading enzymes [281].

In addition, AEA uptake can also occur via a protein-carrier-mediated caveolae-related endocytosis. This process proposes that AEA binds a carrier protein located within the caveolae, a caveolae-derived vesicle (clathrin-independent) is formed and, subsequently, AEA endocytosis occurs; this process is rapid, saturable and energy independent [282]. In this way, the eCBs uptake is still poorly understood and more information about this process is required. Figure 10 resumes the proposed pathways of AEA transport across cell membrane and a model of intracellular trafficking of AEA.

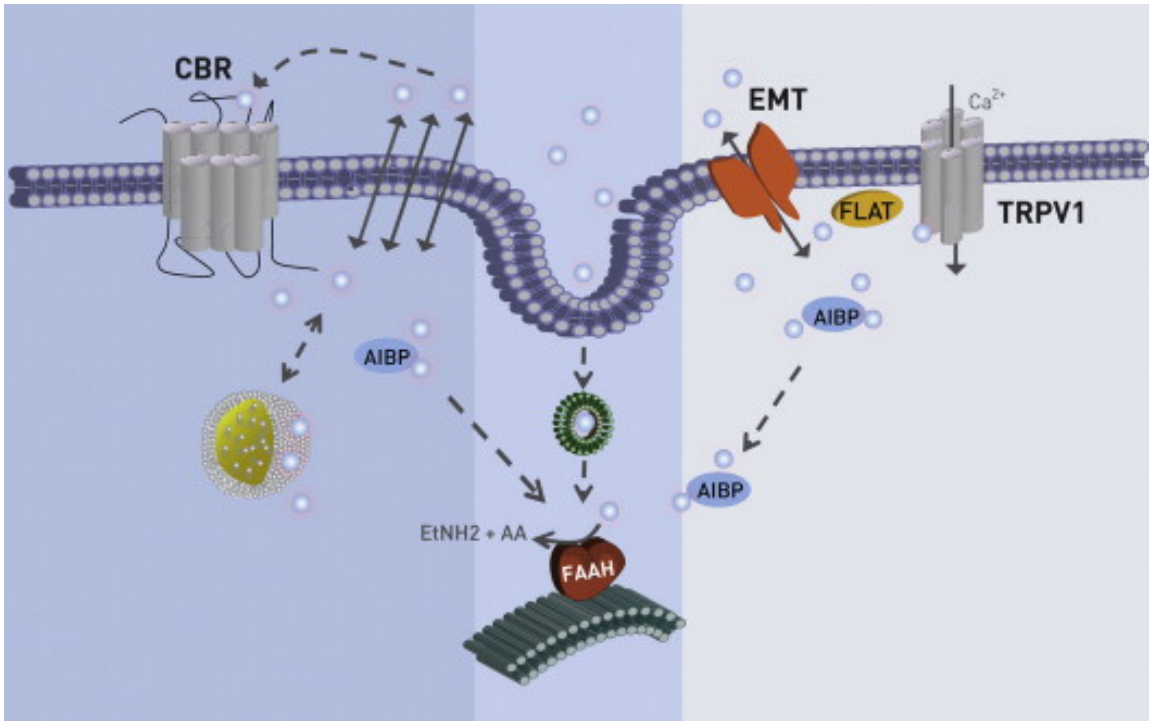


Figure 10. The proposed models for AEA uptake and intracellular traffic.
After activating cannabinoid receptors in extracellular environment (CBR), AEA can passively diffuse across cellular membrane due to a concentration gradient (represented at left, in dark blue). AEA may be transported into the cells via a protein carrier-mediated caveolae-related endocytosis (represented in centre, in blue). Endocannabinoid Membrane Transporter (EMT) may facilitate the internalization of AEA (represented at right, in light blue). AEA may be sequestered inside the cell by binding to AEA Intracellular Binding Proteins (AIBPs) or stored in adiposomes, which may also interact with AIBPs. Moreover, FAAH-like AEA transporter (FLAT) may also participate in AEA uptake. All these proteins may distribute AEA throughout the cytoplasm and cell organelles, facilitating its degradation by fatty acid amide hydrolase (FAAH). Inside the cell, AEA can also activate the transient receptor potential vanilloid 1 (TRPV1) or nuclear receptors [134].

The majority of these models were suggested after studies of AEA transport however, there are evidences that suggest that AEA and 2-AG are accumulated in cells via common mechanisms. Moreover, 2-AG inhibits AEA cellular uptake indicating a competitive nature of the transport of these two eCBs [271].

2.4 Endocannabinoid Signalling

Cannabinoid receptors are expressed in several cells and tissues in the organism. Along with their agonists, CB receptors are crucial for the transduction of endocannabinoid signalling, which modulates multiple cellular pathways in different physiological and pathophysiological mechanisms. The signalling pathways affected after CB receptors activation are summarized in Figure 11.

CB receptors are generally coupled to $G_{i/0}$ proteins, triggering several cellular mechanisms, like AC inhibition, MAPK activation, activation of inward rectifying K^+ (K_{ir}) channels or inhibition of voltage-gated Ca^{2+} channels [283, 284]. The coupling of CB1/2-$G_{i/0}$ was demonstrated by the impairment of these effects after treatment with PTX, a specific inhibitor of $G_{i/0}$ proteins [186, 283, 285]. Nevertheless, under conditions of PTX treatment, CB1 but not CB2 may interact with G_s [286] and $G_{q/11}$ [287] proteins leading to different cellular effects.

The inhibition of some AC isoforms by $G_{i/0}$ proteins-coupled to CB receptors results in a decrease of cAMP levels, a second messenger that stimulates the activity of Protein Kinase A (PKA). Consequently, reduced PKA activity impacts important cellular signalling events including voltage-dependent current flow at A-type K^+ channels [288] and focal adhesion kinase (FAK) phosphorylation [289]. Besides $G_{i/0}$, CB1 may also be coupled to G_s proteins [290]. Recently, it has been described that CB receptors activation induces the phosphorylation of cAMP response element binding protein (CREB) [291, 292].

MAPK pathway regulates cellular functions such as proliferation, differentiation and apoptosis. CB1 and CB2 activation increases the phosphorylation of p38 [293-297], ERK 1/2 [298-302] and c-Jun *N*-Terminal Kinases (JNK) [296, 303, 304]. Moreover, CB receptors activation enhances [305, 306] or inhibits the Phosphoinositide 3-kinase (PI3K)/ Protein Kinase B (PKB or Akt) [307].

Cannabinoid agonists regulate ionic channels through activation of CB1. In fact, they inhibit voltage-gated calcium channels (VGCC) of most types including P/Q, N and L-type channels by a mechanism mediated by $G_{i/0}$ proteins [308-310]. T-type Ca^{2+} channels are also inhibited by AEA through direct binding [311]. Furthermore, CB1 activation promotes the stimulation of K_{ir} channels, also in a $G_{i/0}$-dependent way [312]. TWIK-related acid-sensitive K^+ channels 1 (TASK-1), which are responsible for setting membrane potential, are inhibited by AEA and other CB agonists [313]. CB receptors activation may also increase intracellular Ca^{2+} concentrations through different pathways that aims the release of Ca^{2+} from IP3-sensitive stores [314-316].

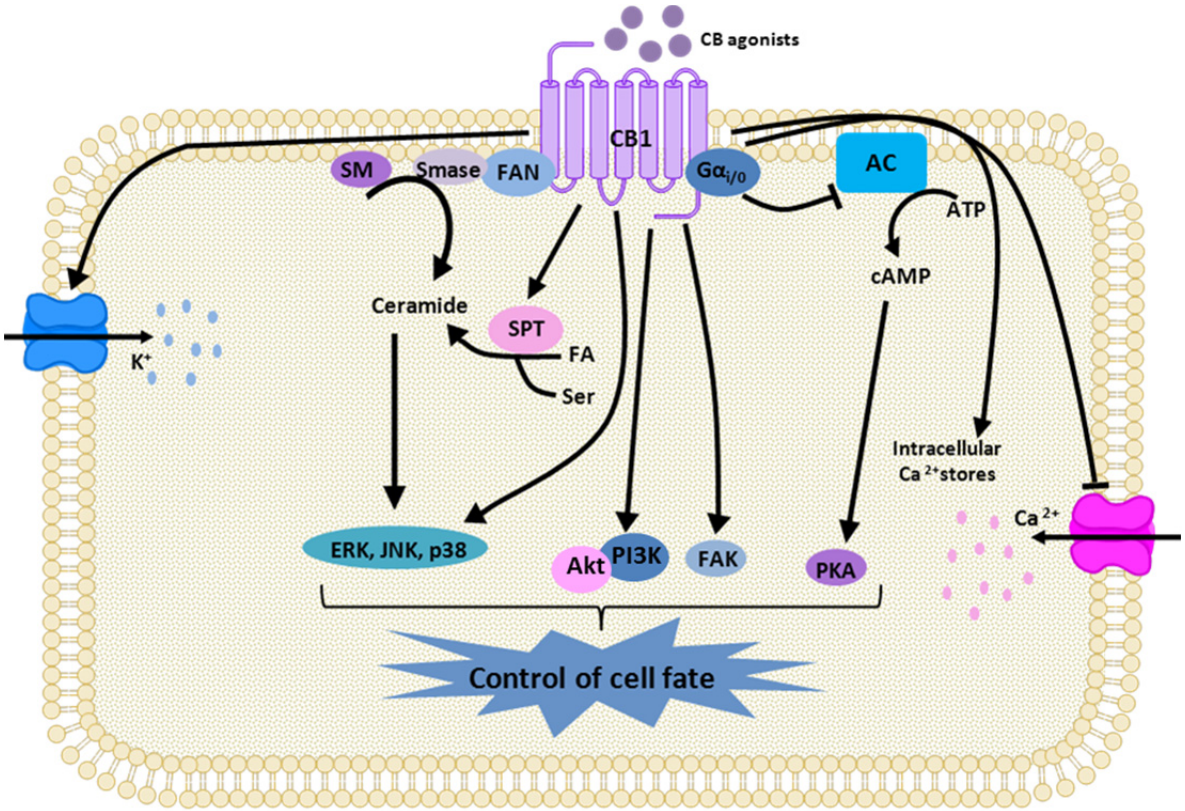


Figure 11. Cannabinoid signalling pathways resulting from the activation of CB receptors coupled to G protein.
The inhibition of AC induces a decrease in cAMP levels, leading to a decrease in PKA activity. CB1 also mediates the stimulation of intracellular kinases such as ERK, JNK, p38, FAK and PI3K/AKT, which are involved in several intracellular mechanisms. Ionic channels can also be regulated by cannabinoid signalling receptors: Ca^{2+} channels inhibited whereas K^{+} channels are induced after the activation of CB1. Moreover, the intracellular levels of Ca^{2+} are increased. The binding of the adaptor protein FAN to CB1 induces ceramide accumulation through the stimulation of SM hydrolysis by SMase. Also, CB1 can stimulate *de novo* synthesis of ceramide by SPT, from FA and Ser. AC-Adenylyl Cyclase; ERK- Extracellular signal-regulated kinase; FA- fatty acid; FAK- focal adhesion kinase; FAN- factor associated with neutral sphingomyelinase activation; JNK- c- Jun N-Terminal Kinases; PI3K-Phosphoinositide 3-kinase; PKA-Protein Kinase A; Ser- serine; SM- sphingomyelin; SMase-Sphingomyelinase; SPT- serinoylpalmitoyltransferase.

The adaptor protein factor associated with neutral sphingomyelinase activation (FAN) is also able to interact with CB1, mediating its interaction with sphingomyelinase (SMase), the enzyme that hydrolysis sphingosine into ceramide. In this way, CB1 triggers an acute generation of ceramide, which is related to the regulation of metabolic functions. Nevertheless, activation of CB1 also induces a long term ceramide accumulation due to the stimulation of its *de novo* synthesis by serine palmitoyltransferase [317]. Ceramide mediates CB1-induced apoptosis in several cell types [297, 318-321]. Besides CB1, CB2 also stimulates ceramide synthesis, inducing apoptosis [155, 321].

Recently, some proteins that specifically interact with CB1 were discovered. CB1 receptor interacting proteins, CRIP1a and CRIP1b, bind to CB1 C-terminal but, till now, only CRP1a effects on modulation of CB1 activity were recognized [322]. G protein-

coupled receptor associated sorting protein 1 (GASP1) interacts with CB1 receptor, inducing its downregulation and regulating its post-endocytic targeting to lysosome [323]. β-arrestins are also involved in desensitization of CB1 receptor, since they bind to agonist-occupied CB1, preventing the signal transduction and initiating the endocytosis of CB1-arrestin complex [324]. Since some of these proteins reside within or depend on lipid rafts, it is possible that the disruption of these structures could negatively influence CB1 desensitization, exacerbating CB1 signalling [325].

2.5 Endocannabinoid system in cell death

Cell death is a cellular event that is crucial for the homeostasis of tissues and organs, while unbalanced cell death rates are associated with several pathological conditions such as cancer, neurodegenerative diseases and pregnancy-related complications. The ECS has emerged as an important intervener in cell death processes like apoptosis, necrosis or autophagy.

Apoptosis is a programmed cell death mechanism by which cells die without damaging the neighbouring cells. This is an ATP-dependent process and occurs mainly by two major apoptotic pathways, the mitochondrial and the death receptor pathways. Cells undergoing apoptosis own morphological characteristic features such as chromatin condensation and fragmentation and membrane blebbing. The pro-apoptotic effects of AEA have been described in several cell types, through CB receptor-dependent and independent mechanisms, triggering different downstream signalling pathways. In fact, after binding to CB1 or CB2, AEA induces apoptosis by activation of caspase-9, loss of $\Delta\psi m$, oxidative stress and ceramide accumulation [297], as well as, cytochrome *c* release [155, 192, 326] or intracellular Ca^{2+} levels increase [327]. Also, AEA-induced apoptosis may require the phosphorylation of p38, ERK1/2 or JNK [297, 326-329], which may occur directly after CB1 activation or ceramide accumulation. Besides AEA, 2-AG can also induce apoptosis in some cell types, such as decidual cells [175] and hepatic stellate cells [178], through the activation of mitochondrial pathway.

CB1 and CB2 activation may also trigger an apoptotic mechanism mediated by the endoplasmic reticulum (ER) stress. In fact, in human pancreatic tumor cells, a CB2 receptor-dependent accumulation of *de novo* synthetized ceramide upregulates the stress-regulated protein p8, the ER stress-related genes activating transcription factor 4 (ATF-4) and telomere repeat binding factor 3 (TRIB-3) [330].

Most of the abovementioned apoptotic mechanisms involve the activation of the mitochondrial pathway. Nevertheless, eCBs may also trigger the death receptor pathway. In fact, in cholangiocarcinoma cells, AEA causes apoptosis through a CB receptor-independent mechanism, by accumulation of ceramide and recruitment of Fas and FasL into lipid rafts [331] and recently, these effects were attributed to the activation of GPR55 [332]. In Chang liver cells, AEA mediates cell death by activation of both extrinsic and intrinsic pathways, with upregulation of FasL, Bim and Bax [333]. The CB2 agonist JWH-015 and THC also induce apoptosis in immune cells, through a crosstalk between extrinsic and intrinsic pathways with activation of caspases-3,-8 and -9 and loss of $\Delta\psi m$ [334, 335]. In addition, in chondrocytes, Gomez et al. reported that AEA induces apoptosis through a CB receptor-independent mechanism that also involves an interplay between intrinsic and extrinsic pathways [154]. In addition, the activation of TRPV1 by AEA triggers the mitochondrial pathway in human neuroblastoma and lymphoma cells [336] and, in human endothelial cells, AEA-induced activation of TRPV1 leads to apoptosis by a mechanism dependent on p38 and JNK phosphorylation [337].

A role for COX-2 oxygenated metabolites of AEA, the prostamides, in cell apoptosis has recently emerged. In keratinocytes, AEA mediates apoptosis after being metabolized to J-series prostaglandins, which generate oxidative stress [250]. Moreover, the AEA-induced cell death in colon cancer cells is also dependent on COX-2 but independent on oxidative stress [249]. Recently, in melanoma cells, it was reported that AEA proapoptotic effects are, at least partially, mediated by COX-2 and also LOX metabolites [338].

The eCBs have also been associated with necrosis. In fact, AEA and 2-AG induce necrosis in rat decidual cells [138, 175]. AEA induces necrosis in hepatic stellate cells, by increasing intracellular Ca^{2+} levels and oxidative stress [339] and through the downregulation of the prosurvival pathway PI3K/Akt and induction of inflammatory cytokines production [202].

Autophagy is a cellular event pivotal for cell homeostasis that is involved in both cell survival and death. It consists in enclosing cellular components into autophagosomes (double-membrane vesicle), which will fuse with lysosomes to be degraded and recycled. Recently, new data have reported the involvement of the endocannabinoid signalling in autophagy-related apoptosis. In fact, THC stimulates *de novo* synthesis of ceramide via CB1 in glioma cells, initiating an ER stress response, which induces autophagy via TRIB3-dependent inhibition of Akt/mTORC1 (mammalian target of rapamycin complex 1) axis, a repressor of this cellular event. Moreover, it was reported that autophagy is required for the cannabinoid antitumoral action, since it was crucial for the activation of the

mitochondrial pro-apoptotic pathway [340]. In hepatocellular carcinoma, THC and CB2 agonist JWH-015 induce an autophagic process that is also important for cell apoptosis. Here, autophagy is induced either by activation of adenosine monophosphate-activated kinase (AMPK) or by ceramide accumulation. The latter leads to TRIB3 activation, inhibiting Akt/mTORC axis and activating PPAR-γ, which leads to autophagy and then to cell apoptosis [341, 342].

In addition to the aforementioned cell death mechanisms, in some types of mantle lymphoma cells, the CB1 agonist WIN55-212,2 induces paraptosis, a non-apoptotic programmed cell death mechanism characterized by the presence of cytoplasmic vacuoles of ER origin, which differ from the autophagic vacuoles, since they do not fuse with lysosomes [343].

In summary, the participation of endocannabinoid signalling in different cell death processes seems to be cell specific, since it may trigger different cell death pathways and interfere with several cellular signalling mechanisms.

3. Endocannabinoids in reproduction

The hazards of *Cannabis sativa* in reproductive function are known for years. Its major psychoactive component, THC, induces a decline in pituitary hormones, follicle stimulating hormone (FSH), luteinizing hormone (LH) and prolactin, and also in sex hormones, progesterone, oestrogens, and androgens, having a negative outcome in fertility. THC also inhibits ovulation, crosses placenta and can accumulate in maternal milk [344-346]. The consumption of cannabis during gestation is implicated in deficient foetal growth, low birth weight, preterm labour and long-term neurobehavioral disturbances [347, 348]. With the discovery and identification of CB receptors and their endogenous compounds that are capable of mimicking the THC effects, an interest in the study of their implications in physiologic and pathophysiologic mechanisms has emerged. Also, CB receptors are present in female and male reproductive organs and tissues like ovaries, uterus, placenta, testis, prostate, sperm and embryo. Thus, ECS has been a target for the study of male and female fertility and of different stages of pregnancy like preimplantation, implantation, decidualization, placentation, foetal development and labour. Figure 12 summarizes eCBs effects on these reproductive events.

3.1 Endocannabinoid system in female reproductive tract and fertility

The presence of eCBs in several female reproductive tissues, organs and fluids suggests a role for this system in physiological reproductive processes. AEA is present in mid-cycle oviductal fluid, follicular fluid, amniotic fluid, milk and in human seminal plasma, suggesting a role for AEA in modulation of multiple physiological and pathophysiological processes, such as follicular maturation, ovulation, placental and foetal development and lactation.

Human ovaries express both cannabinoid receptors in cortex and medulla and CB2 expression is generally higher. FAAH and NAPE-PLD are expressed only in secondary and tertiary follicles, corpus luteum and corpus albicans, which enable the AEA synthesis in the ovaries [349]. Peralta *et al.* recently demonstrated that human oocytes express CB1 and CB2 and that their localisation change during the various stages of meiotic resumption [350]. During the menstrual cycle, plasmatic AEA levels suffer alterations, peaking in the early follicular phase [351]. Moreover, the plasmatic peak of AEA is achieved at the ovulation and, then, AEA levels decrease in early luteal phase [352].

During menstrual cycle, it is also verified a correlation between AEA levels and oestradiol, FSH and LH suggesting that these hormones may have a role in regulation of this eCB [352]. In the periovulatory phase, while progesterone synthesis is supressed, the lowest levels of this hormone coincide with lowest levels of FAAH. Also, both molecules reach maximal values at luteal phase. [353]. Together, these data suggest a role for endocannabinoid system in folliculogenesis, follicle and oocyte maturation and ovulation.

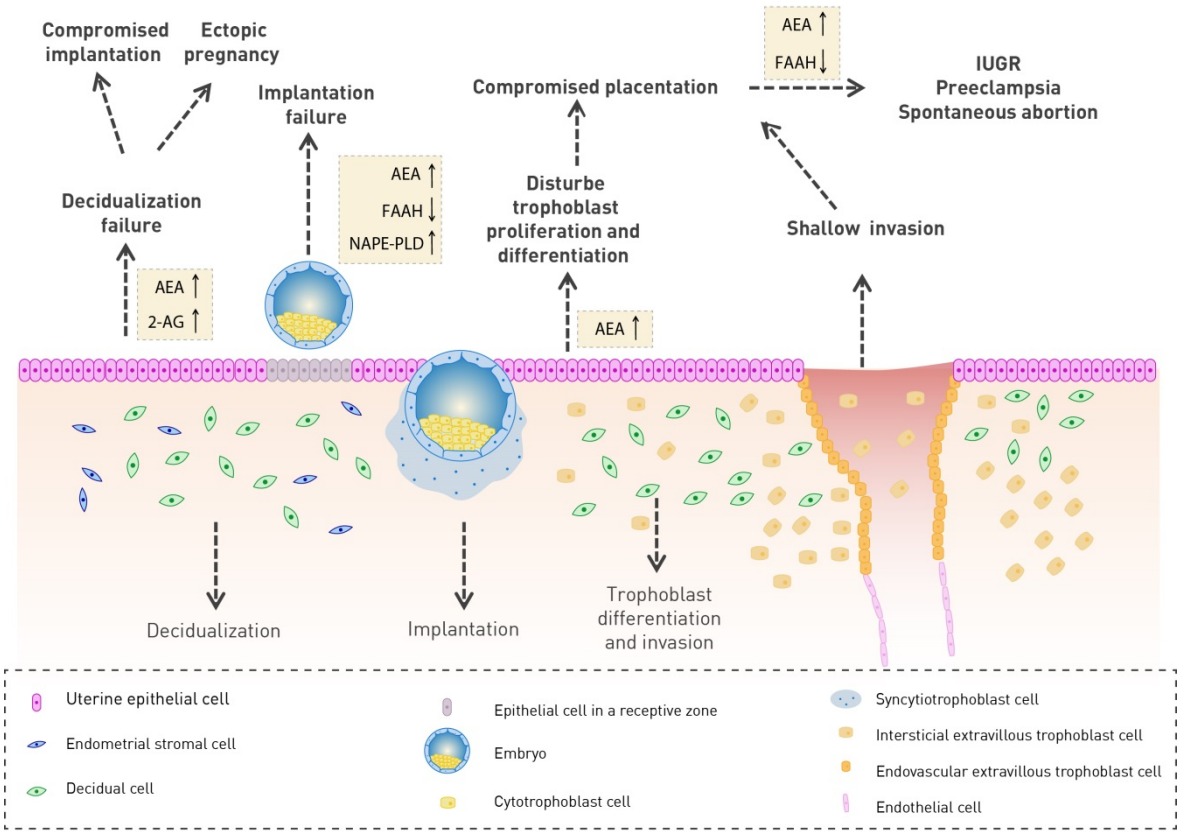


Figure 12. Schematic representation of endocannabinoids (eCBs) effects throughout pregnancy and the negative impact of an abnormal endocannabinoid signalling.
Decidual cells result from the differentiation of endometrial stromal cells, in order to turn the uterus receptive to the implanting embryo. Trophoblast cells proliferate, differentiate, invade maternal tissues and undergo apoptosis, allowing the placental development. Emerging data indicate that eCBs are part of the complex network of hormones, cytokines and other molecules that regulate the reproductive events, namely implantation and decidualization. Furthermore, it is suggested that an abnormal endocannabinoid signalling (yellow boxes) may be involved in the pathophysiology of pregnancy-associated complications, such as intrauterine growth restriction, preeclampsia and spontaneous abortion [354].

The members of ECS are also expressed in human uterus, during menstrual cycle. CB1 and CB2 receptors are widely distributed in uterine tissues. CB1 is expressed with higher intensity in endometrial glands than in stromal cells, whereas CB2 receptor immunoreactivity is minimal at the beginning of a new cycle, increasing till its maximal expression in late-proliferative phase. Moreover, it was verified an increase of FAAH and

a decrease in NAPE-PLD expression during menstrual cycle in endometrium till mid-secretory phase [355]. Recently, Scotchie *et al.* corroborated some of these findings reporting that FAAH expression was enhanced in secretory phase but they also reported augmented NAPE-PLD expression. Moreover, they described an increased expression of MAGL and COX-2 in this phase, supporting that low levels of 2-AG may also be required during the implantation period [356].

The eCBs seem also to have a role in the regulation of human myometrial function. In fact, in the human myometrial smooth muscle cell line ULTR, AEA activates ERK1/2 pathway in a CB1-dependent mechanism and inhibits AC, inducing cell viability loss [357]. Recent data, in human myometrium, reported that NAPE-PLD, FAAH and CB1 are expressed but TRPV1 and CB2 are absent in this tissue [358].

In endometrial stromal cells, the non-hydrolysable AEA analogue methananadamide induces cell migration via CB1-dependent activation of PI3K/Akt and ERK1/2 pathways [359]. Moreover, methanandamide enhances ESC proliferation and, longer exposures, induces apoptosis [360].

During human ovulatory cycle, there are oscillations in the levels of AEA and FAAH in peripheral lymphocytes. The expression of NAPE-PLD, EMT and CB receptors remain constant during the cycle [353]. The highest FAAH levels and the concomitant lowest AEA levels are found in the luteal phase, suggesting that these levels may be important for a successful implantation [353, 361].

The evidences of interplay of sex steroids, cytokines and eCBs result essentially from the modulation of FAAH, the major controlling enzyme of AEA, by oestrogen, progesterone, leptin and Th cytokines, indicating a role for eCBs-hormone-cytokines in the regulation of human fertility [362]. Maccarrone *et al.* demonstrated that progesterone stimulates FAAH activity in human lymphocytes [363]. In addition, profertility Th2 cytokines (IL4, IL10) stimulate FAAH activity whereas the antifertility Th1 cytokines (IL2, IL12 and interferon γ) have the opposite effect [363]. It was already verified that the activation of FAAH by progesterone results from an increase of nuclear levels of transcription factor Ikaros, which binds to FAAH promoter, inducing *FAAH* gene expression [364]. The adipokine leptin also induces activation of FAAH in human T lymphocytes through the activation of signal transducer and activator of transcription 3 (STAT3), which upregulates a cAMP response element (CRE)-like site in FAAH promoter [365]. Thus, leptin and progesterone seem to exert a synergic effect in FAAH upregulation and consequently in the modulation of AEA levels. Furthermore, leptin and progesterone upregulate FAAH activity in human lymphoma cell line U937 [366]. Moreover, since they

decrease AEA levels, leptin and progesterone have a protective role in immune cells through the inhibition of AEA pro-apoptotic effects [367]. In addition, in the uterus of leptin knockout mice, AEA and 2-AG levels are elevated comparatively with wild-type mice, due to the decreased FAAH and MAGL activities and higher activity of DAGL [368]. Together, the data suggest that leptin and progesterone may interfere with the endocannabinoid signalling, reinforcing that abnormal levels of these hormones may be implicated in infertility. Figure 13 schematises the network in human lymphocytes, where FAAH has a central role.

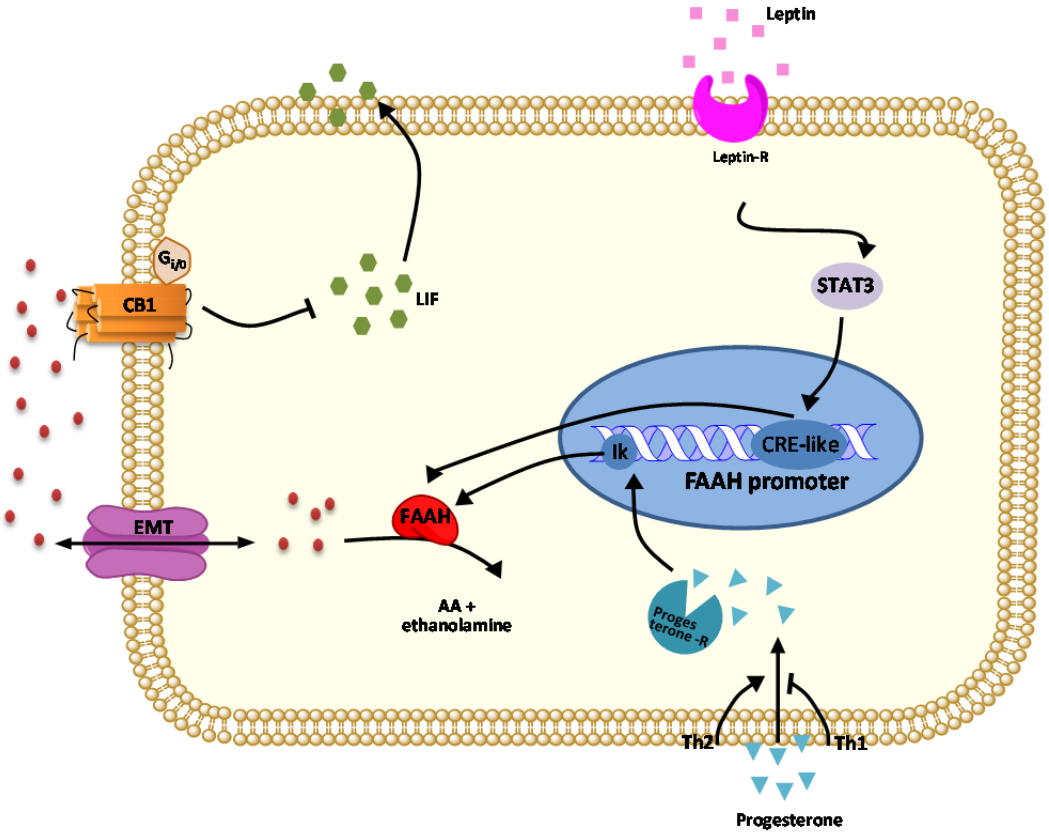


Figure 13. Role of Endocannabinoid system in hormone-cytokine network.
After its biosynthesis, AEA is released in the intracellular space, where it activates CB1 and, among other actions, inhibits LIF release. AEA is transported into the cell via the putative EMT and is hydrolysed by FAAH to AA and ethanolamine. FAAH is upregulated by a synergic process mediated by progesterone and leptin. Progesterone binds to its intracellular receptor and upregulates the FAAH promotor through the transcription factor Ikaros (Ik). Leptin activates its membrane receptor, leading to the activation of STAT3, which will bind to CRE-like site, activating the transcription of *FAAH* gene. Moreover, FAAH activation by progesterone is enhanced by Th2 cytokines and inhibited by Th1 cytokines. AA- arachidonic acid; AEA- anandamide; CRE- cAMP response element; EMT- endocannabinoid membrane transporter; FAAH- fatty acid amide hydrolase; Ik- Ikaros; LIF-leukemia inhibitory factor; STAT3- signal transducer and activator of transcription 3.

Leukemia Inhibitory Factor (LIF) is a member of IL-6 cytokine family produced by T lymphocytes, which is important for a successful pregnancy. Indeed, LIF promotes embryo implantation and survival, is involved in the decidualization process and stimulates hCG production by trophoblasts [369]. Uterine levels of AEA are higher in LIF-deficient mice [370]. Furthermore, AEA and 2-AG decrease the release of LIF through activation of CB1 and so, low FAAH activity may negatively impact the pregnancy outcome due to the reduction of LIF levels [363].

3.2 Preimplantation

Preimplantation is the reproductive event where the embryo begins its development. Mouse uterus and oviduct express the enzymatic machinery necessary for the synthesis of AEA [371, 372]. The preimplantation mouse embryo expresses mRNA of both CB receptors. *In vitro*, CB1 receptor agonists arrest the development of 2-cell embryos, inhibit the compaction and reduced the trophectoderm cells number [373, 374]. Moreover, the development of preimplantation embryo became asynchronous in CB1 or CB2 receptor deficient mice [370]. Recent evidences support that high levels of methanandamide, a CB1 agonist, induce autophagic activation and apoptosis in mouse blastocyst [375]. All these results suggest that deregulated endocannabinoid signalling via CB1 receptor impairs preimplantation process and consequently pregnancy course.

The embryo transport along the oviduct to the uterus is achieved by a sequence of controlled contractions and relaxations of oviduct smooth muscle. Alterations in expression of CB1 receptor impairs the coordinated muscle contraction and relaxation, inducing embryo retention and possibly leading to implantation failure in mice [376]. In mouse oviduct, a balance between the expression of NAPE-PLD and FAAH is required to create locally an appropriate “anandamide tone” for normal development of embryos and their oviduct transport. NAPE-PLD is present in embryos during preimplantation period, since fertilization till the blastocyst stage, while FAAH is first expressed in 2-cell embryos. In the oviduct, it was verified an inverse distribution of these enzymes on days 1-4 of pregnancy: NAPE-PLD is expressed in higher levels in epithelium of the isthmus than of the ampulla, while FAAH levels are higher in the ampulla epithelium than in the isthmus. Furthermore, FAAH inactivation and the concomitant high AEA levels induce a retarded development, oviduct retention of embryos and impaired implantation through CB1 receptor activation [377]. This suggests that FAAH is a key regulator of AEA tone and

CB1-activated pathways, which are crucial for the oviductal transport and must be tightly controlled to achieve a successful pregnancy.

The embryo retention in fallopian tubes resulting from a dysfunctional muscle contraction is one of the causes of human ectopic pregnancy. In women with this pregnancy disorder, the expression of CB1 mRNA is lower in fallopian tubes and decidua, in comparison with normal pregnancy [378]. Gobeh *et al*. also reported that AEA levels were increased in ectopic fallopian tubes, whereas CB1 and FAAH expression was decreased [379]. In addition, FAAH activity was decreased in peripheral blood cells of women with ectopic pregnancy and AEA blood levels were increased [380].

3.3 Implantation and embryo development

Implantation is one of the most critical pregnancy events and requires a synchronized interaction between the trophoblast cells of activated blastocyst and the receptive uterus, which allows a suitable attachment of blastocyst. This event should occurs within the period of time where the activated stage of the blastocyst is superimposed on the receptive state of the uterus, the window of implantation [381]. The implantation process is highly regulated and it is proposed a role for ECS in this process. Indeed, in mouse uterus, AEA levels are spatiotemporal regulated during the gestational period. Down-regulated levels of AEA detected at implantation sites are associated with uterine receptivity [382]. Higher f*aah* and low *nape-pld* mRNAs in non-implantation sites correlate with the refractory uterus to embryo implantation and with low FAAH and NAPE-PLA expression [383-385]. Moreover, AEA reduces the zona-hatching of blastocysts and inhibits the implantation through CB1 activation [382]. In contrast, low AEA levels promote blastocyst attachment, accelerate trophoblast differentiation and outgrowth via CB1 receptor activation but higher AEA levels impair these processes [386, 387]. This dual AEA effect results from the activation of two independent signalling pathways. In fact, low AEA concentrations promote blastocyst implantation through ERK phosphorylation in trophectoderm cells, while higher concentrations inhibit the activity of Ca^{2+} channels, hampering the implantation [141]. Moreover, uterine AEA levels and CB1 expression in the blastocyst are down-regulated prior to the implantation in mice, whereas in non-receptive uterus and dormant blastocysts, their levels are higher, supporting a role for the endocannabinoid signalling in embryo synchronization with the uterine receptivity [370]. The other major eCB, 2-AG has the same expression pattern as AEA i.e., lower levels at implantation sites and higher at interimplantation sites, possibly also participating in

regulation of window of implantation [174]. Further, the expression pattern of 2-AG metabolic enzymes was also in agreement with the required low levels of eCBs at implantation sites: low levels of DAGL-α and high MAGL levels. In addition, COX-2 is expressed at implantation sites but is almost undetectable at interimplantation sites, which indicate a contribution of this enzyme in differential regulation of AEA and also points to a role for COX-2 derived prostaglandins in implantation [174].

Mouse blastocysts release a soluble lipid compound that increases the activity but not the expression of uterine FAAH enzyme by 2, 5-fold. The FAAH activator is produced by trophoblasts and ICM and suggests that blastocysts may protect themselves from the toxic effects of high AEA uterine levels [388].

In women that underwent *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICIS)-embryo transfer, it was verified that AEA plasma levels of pregnant women decreased from the day of oocyte retrieval to that of embryo transfer, increasing till 4-5 weeks of gestation and achieving minimal values at 6 weeks. In contrast, in non-pregnant women, the lowest levels of AEA in plasma were registered in the day of oocyte retrieval and the decline till the embryo transfer was not observed [389]. These observations suggest that high levels of AEA in ovulation and lower levels in implantation period are required for a successful pregnancy in humans, as occurs in rodents. Also, during menstrual cycle, AEA plasma levels decreased in early luteal phase, coinciding with the window of implantation and reinforcing that low levels of AEA are beneficial to embryo implantation [352]. In summary, abnormal expression of AEA metabolic enzymes in uterus induce a deregulation of AEA levels that may lead to a deficient implantation and, consequently, to complications during gestation.

3.4 Endocannabinoid system and pregnancy maintenance

The ECS may also have a fundamental role in the maintenance of a healthy pregnancy. The members of the system are expressed throughout gestation in decidua, placenta and myometrium [372, 390-394]. Moreover, the major endocannabinoid AEA is present in human female reproductive tissues, namely in placenta, foetal membranes and also in cord blood, umbilical vein and artery [395]. During the first and second trimesters of human pregnancy, the plasma levels of AEA are comparable to those registered in the luteal phase of menstrual cycle, slightly increasing in third trimester, which is in agreement with the required low levels of AEA in the early stages of pregnancy [351, 361].

Decidua is the maternal tissue that supports the embryo growth and maintains the early pregnancy by protecting the embryo from maternal immune system and participates in gas and nutrients exchanges. In women, decidualization is independent of blastocyst's presence, since it occurs in every luteal phase of menstrual cycle to prepare the uterus for an eventual pregnancy. The formation of decidua and placenta is a highly regulated process that involves a network of hormones and cytokines, where eCBs also seem to play an important role. In fact, the CB1 receptor agonist WIN inhibits human decidualization and induces apoptosis by decreasing cAMP levels, a second messenger that enhances decidualization [396]. Moreover, in primary cultures of rat decidual cells, AEA and 2-AG induce apoptosis by CB1 activation, suggesting a role for ECS in rat decidual regression [138, 175].

The study of CB receptors expression in rat foetoplacental units revealed that CB1 expression is more intense than CB2 and is highest with the maximum decidual development [397]. The levels of AEA and 2-AG in plasma and decidual tissue fluctuate, though plasmatic levels do not correlate with tissue levels, suggesting an *in situ* enzymatic regulation of eCBs levels [175, 398]. Moreover, NAPE-PLD and FAAH activities in maternal tissues increase throughout pregnancy and the NAPE-PLD:FAAH ratio is higher when the decidua reaches its maximal development [399].

In the human placenta, CB receptors expression was described for the first time by Kenney *et al.* in term placentas and in the choriocarcinoma cell line BeWo [400]. Park *et al.* reported that, in term placenta, CB1 was expressed in all tissues while FAAH expression was more diffuse, with highest expression in the decidua and amnion; the ST presents higher FAAH expression than CTs [392]. In first trimester placenta, Heliwell *et al.* observed that FAAH was expressed in EVTs, CTs and ST. Moreover, its expression seems to be modulated during gestation since its mRNA peaks at the week 11, then decreasing till the end of pregnancy [401]. Recently, Habayeb *et al*. analysed the temporal-spatial expression of FAAH and CB receptors during early pregnancy and demonstrated that, besides FAAH, both CB1 and CB2 are also expressed in placenta. In fact, CB1 receptor was expressed in CTs and ST but, in the latter, the signal intensity diminished at 10^{th} week. CB2 receptor expression remained constant during early pregnancy [139]. Moreover, FAAH was also expressed throughout placental tissues but the signal in ST increased gradually, peaking at 10^{th} week of gestation, decreasing thereafter [139]. These data support a role for FAAH in the regulation of placental AEA levels and indicates that this enzyme may play a role in foetal protection against maternal blood AEA, in the initial stages of pregnancy. A recent study demonstrated that, in

preeclamptic placentas, NAPE-PLD was upregulated whereas FAAH was downregulated, in comparison with normal placentas [402], suggesting that deregulations of the ECS may be implicated in the pathophysiological mechanisms of preeclampsia.

In women that underwent spontaneous abortion, high levels of CB1 receptor and very low levels of FAAH were found in 1st trimester placentas, comparatively with placentas of women that underwent voluntary pregnancy termination [403]. The AEA plasma levels of women with recurrent spontaneous miscarriages was about 3-fold higher than those observed in women with successful gestations [404]. Moreover, decreased FAAH levels were detected in lymphocytes of pregnant women that later suffered spontaneous miscarriage [405]. Also, FAAH levels in lymphocytes of women who failed to achieve an ongoing pregnancy after IVF-embryo transfer were lower comparatively with women that became pregnant, and as expected, blood levels of AEA were higher [406]. Due to these reports, low FAAH levels in peripheral lymphocytes have been proposed as an early marker of human spontaneous miscarriage.

Sun *et al.* observed an inhibition of trophoblast stem cells proliferation, differentiation and invasiveness in CB1 knockout mice, causing an abnormal placentation, due to the decrease of AKT phosphorylation [407]. Microarray data reported that silencing or enhancing CB1 signalling in these cells affected the expression of several genes, compromising cell migration [408]. Moreover, AEA inhibits the proliferation of BeWo cells through CB2 receptor [139] and THC decreases BeWo cells proliferation and modulates genes encoding for proteins involved in growth, apoptosis, cell morphology and ion channels [409].

Aims

Placental formation and development requires tightly coordinated processes of trophoblast cells proliferation, apoptosis and differentiation. Although various molecules and signalling pathways have been identified as regulators of these processes, there are still pieces missing to fully understand the complexity of the placental development.

The endocannabinoids have been pointed as new modulators in neuroprotection, apoptosis, pain modulation or inflammation. In the biology of reproduction, a role for the ECS has already been recognized in the processes of decidualization and implantation. Nevertheless, the linkage between these lipids and the human placental development has been poorly explored, though some ECS members (CB1, CB2, FAAH and NAPE-PLD) have already been described in placental tissues. In this way, one of the major aims of this work will be to search for the expression of other members, such as the main 2-AG metabolic enzymes (DAGL-α, MAGL) and of the AEA target, TRPV1, in primary human cytotrophoblasts, syncytiotrophoblast and in the cytotrophoblast cells BeWo, a widely used model of CTs. How the turnover of villous trophoblast is regulated is important for understanding normal and complicated pregnancies. In that way, the impact of endocannabinoids in the processes of cytotrophoblast apoptosis and differentiation and the underlying signalling pathways will be studied. The role of endocannabinoid and endovanniloid signalling during CTs biochemical and morphological differentiation into STs will also be explored by the analysis of the effects on the modulation of placental proteins expression such as hCG, leptin and fusogenic proteins

Together, this work aims to shed light on the role of endocannabinoids in cytotrophoblast cell turnover, and the cellular mechanisms behind their effects. These studies may contribute to identify novel modulators of cytotrophoblast remodelling and, consequently, of placental development and also to unravel the involvement of deregulations of ECS in the pathophysiology of some pregnancy-related complications.

PART II

Experimental section

Manuscript I

2-arachidonoylglycerol in cytotrophoblasts: Metabolic enzymes expression and apoptosis in BeWo cells.

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Reprinted from Reproduction, 2014; 147:301-11. doi: 10.1530/REP-13-0563.

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RESEARCH

2-Arachidonoylglycerol effects in cytotrophoblasts: metabolic enzymes expression and apoptosis in BeWo cells

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Abstract

The major endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG) is a member of the endocannabinoid system (ECS) that participates in cell proliferation and apoptosis, important events for the homoeostasis of biological systems. The formation of placenta is one of the most important stages of pregnancy and its development requires highly regulated proliferation, differentiation and apoptosis of trophoblasts. Anomalies in these processes are associated with gestational pathologies. In this work, we aimed to study the involvement of 2-AG in cytotrophoblast cell turnover. We found that 2-AG biosynthetic (diacylglycerol lipase A) and degradative (monoacylglycerol lipase) enzymes are expressed in human cytotrophoblasts and in BeWo cells. We also found that 2-AG induces a decrease in cell viability in a time- and concentration-dependent manner and exerts antiproliferative effects. The loss of cell viability induced by a 48-h treatment with 2-AG (10 μM) was accompanied by chromatin fragmentation and condensation, morphological features of apoptosis. Additionally, 2-AG induced an increase in caspase 3/7 and 9 activities, a loss of mitochondrial membrane potential (Δψm) and an increase in reactive oxygen species (ROS)/reactive nitrogen species (RNS) generation, suggesting the activation of the mitochondrial pathway. Moreover, whereas Δψm loss and ROS/RNS generation were significantly attenuated by the antagonists of both the cannabinoid receptors 1 and 2 (CB1 and CB2), the increase in caspase 3/7 and 9 activities and loss of cell viability were reversed only by the antagonist of CB2 receptor; the blockage of the eCB membrane transporter and the depletion of cholesterol failed to reverse the effects of 2-AG. Therefore, this work supports the importance of cannabinoid signalling during cytotrophoblast cell turnover and that its deregulation may be responsible for altered placental development and poor pregnancy outcomes.

Reproduction (2014) **147** 301–311

Introduction

The endocannabinoid system (ECS) is present in several tissues and organs and constitutes a novel target in several physiological and pathophysiological situations. The actual members of the ECS are the two cannabinoid receptors CB1 and CB2 and their endogenous ligands (endocannabinoids (eCBs)) and the enzymes involved in the biosynthesis and degradation of eCBs and their putative membrane transporter. There are several endogenous ligands of CB receptors and anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are considered to be the 'major' eCBs. There are also some other G-protein-coupled receptors that have been described as targets for eCBs (see Fonseca *et al.* (2013) for a review).

2-AG is synthesized from membrane phospholipids, mainly through a reaction catalysed by diacylglycerol lipase (DAGL) and it is degraded in the cytoplasm into arachidonic acid and glycerol by monoacylglycerol lipase (MAGL). This eCB acts as a full agonist of both CB1 and CB2 receptors, whereas AEA is only a partial agonist (Pertwee *et al.* 2010), which leads some authors to defend that 2-AG is the 'true eCB' (Sugiura 2009).

2-AG has been pointed out as an important messenger in multiple physiological processes in the nervous, immune and cardiovascular systems, though its exact function still remains unclear (Sugiura 2009). It has been reported that this eCB induces apoptosis in some cell types such as decidual cells (Fonseca *et al.* 2010) and hepatic stellate cells (Siegmund *et al.* 2007). On the other hand, 2-AG is also involved in pro-proliferative effects in cholangiocarcinoma cells (DeMorrow *et al.* 2007) and neuroprotection (Chen *et al.* 2011). The eCBs may play a role in the network of lipids, proteins, cytokines and hormones that regulates the gestational events. Thus, its homoeostasis seems to be crucial for a proper pregnancy outcome. However, little information is available regarding the role of 2-AG in fetal–placental development. In rats, the main metabolic enzymes of 2-AG are expressed in the decidua throughout the gestation, whereas in the placenta they are expressed

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0563
Online version via www.reproduction-online.org

from mid-pregnancy to term pregnancy (Fonseca *et al.* 2012). Moreover, 2-AG induces apoptosis by the activation of CB1 receptor, which indicates a role for this eCB in decidual development and regression (Fonseca *et al.* 2010). Nevertheless, the involvement of this molecule in human placentation is not clear, though some members of the ECS in this organ have been described. In fact, CB1 and CB2 receptors and the main metabolic enzymes of AEA, fatty acid amide hydrolase (FAAH) and *N*-arachidonoylphosphatidylethanolamine-phospholipase D (NAPE-PLD), have been described to be present in first-trimester and term placentas, as well as in BeWo cells (Kenney *et al.* 1999, Park *et al.* 2003, Helliwell *et al.* 2004, Habayeb *et al.* 2008, Trabucco *et al.* 2009, Taylor *et al.* 2011). Furthermore, AEA induces a decrease in the viability of BeWo cells, a cell model of cytotrophoblasts, via CB2 receptor (Habayeb *et al.* 2008), suggesting a role for the ECS during placentation.

The formation of placenta involves continuous tissue remodelling; apoptosis is a crucial cellular event during this process. In fact, cytotrophoblast cell turnover is very proactive during gestational time: cytotrophoblasts proliferate and differentiate into multinucleated cells named syncytiotrophoblasts, while the quiescent cells die by apoptosis. Alterations in the apoptotic rates during pregnancy have been widely reported to be related to some gestational complications such as pre-eclampsia, intrauterine growth restriction (IUGR) and gestational trophoblastic disease (Smith *et al.* 1997, Wong *et al.* 1999, Allaire *et al.* 2000, Leung *et al.* 2001, Crocker *et al.* 2003, Roje *et al.* 2011). To highlight the role of 2-AG in cytotrophoblast cell turnover occurring during the development of placenta, this work aimed to investigate the expression of the main metabolic enzymes of 2-AG (DAGLA and MAGL) in human cytotrophoblasts and in a cytotrophoblast *in vitro* cell model as well as the involvement of 2-AG in trophoblast cell proliferation and death. In addition, the signalling pathways activated by this eCB were studied. In these studies, we assessed the presence of 2-AG metabolic enzymes in primary human cytotrophoblasts and in BeWo cells. The choriocarcinoma cell line BeWo was considered a good model for this work, as BeWo cells have the ability to proliferate and produce hormones and proteins similar to human cytotrophoblasts, express CB receptors and other members of the ECS and respond to cannabinoid stimulation (Habayeb *et al.* 2008). As primary cultures of cytotrophoblasts spontaneously differentiate *in vitro* into syncytiotrophoblasts (Kliman *et al.* 1986), the BeWo cell line was used to study the effect of 2-AG on cytotrophoblast cell turnover.

Materials and methods

Materials

The human choriocarcinoma cell line BeWo was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). DMEM/F12 medium, fetal bovine serum (FBS), antibiotic–antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B), 2.5% trypsin, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and TRIzol reagent were from Gibco/Invitrogen Corporation. Rabbit antibodies anti-MAGL, anti-DAGLA and β-tubulin and rabbit IgG were from Santa Cruz Biotechnology. The peroxidase-conjugated goat anti-rabbit secondary antibody and the Vectastain ABC Rabbit Kit were from Vector Laboratories, Burlingame, CA, USA. Nitrocellulose membranes and Percoll were from GE Healthcare, Chalfont St Giles, Bucks, UK. The Super Signal West Pico Chemiluminescence Detection Kit was from Pierce, Rockford, IL, USA, and X-ray films were from Kodak XAR, Eastman Kodak. DNAse, paraformaldehyde, Sigma Fast Red tablets, Mayer's haematoxylin solution, protease inhibitor cocktail, Triton X-100, methylthiazolyldiphenyl-tetrazolium bromide (MTT), Höechst 33342, methyl-β-cyclodextrin (MCD), *N*-acetylcysteine (NAC), staurosporine (STS), carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA) and H_2O_2 were from Sigma–Aldrich Co. Ethanol, isopropanol and methanol were from Fisher Scientific, Loughborough, UK. DMSO was from VWR, Fontenay-sous-Bois, France. Z-VAD-FMK was from BD PharMingen, San Diego, CA, USA. Aquamount medium was from BDH Laboratory Supplies, Poole, England. The iScript Select cDNA Synthesis Kit and the Experion RNA StdSens Kit were from Bio-Rad Laboratories. The KAPA SYBR FAST qPCR Master Mix 2× Kit was from Kapa Biosystems, Woburn, MA, USA. 2-AG, AM251, AM630 and AM404 were from Tocris Bioscience, Bristol, UK. CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit, Caspase-Glo 3/7 and Caspase-Glo 9 were from Promega. ^{3}H-thymidine was from Amersham. Giemsa was from Merck. Ninety-six-well white plates were from Thermo Scientific, Roskilde, Denmark. Ninety-six-well black plates and eight-well chamber slides were from BD Biosciences, Erembodegem, Belgium. Brains were collected from Wistar rats obtained from Charles River Laboratories, Barcelona, Spain, and all the procedures were conducted in accordance with the guidelines of the Ethics Committee of the Institute of Molecular and Cellular Biology, Porto, Portugal.

Primary cultures of human cytotrophoblasts

Term placentas of normal pregnancies were immediately collected after spontaneous delivery or elective caesarean section. All the procedures were conducted in accordance with the Ethical Committee of Hospital S. João, Porto. Cytotrophoblast cells were isolated as described previously (Kliman *et al.* 1986, Keating *et al.* 2007). Briefly, placentas were washed with saline solution to remove the blood, and villous tissue was dissected and digested in a solution of trypsin and DNAseI. Then, the resulting cells were separated in a discontinuous Percoll gradient. Cytotrophoblasts were collected and seeded in 21 cm^2 dishes or eight-well chamber slides at densities 1×10^7 or 4.5×10^5 respectively. The cells were incubated in DMEM/F12 medium supplemented with 10% (v/v) of FBS and an antibiotic–antimycotic solution at 37 °C in 95% air/5% CO_2 humidified atmosphere. After adherence, the cells were washed with PBS and processed as described below.

The purity of the cytotrophoblast cell culture was assessed by immunocytochemistry with anti-vimentin and anti-cytokeratin antibodies using cells fixed with 4% paraformaldehyde. Culture was considered pure when 95% or a higher percentage of cells were cytokeratin positive.

BeWo cell culture

BeWo cells were maintained in DMEM/F12 medium supplemented with 10% (v/v) of FBS and an antibiotic–antimycotic solution and were incubated at 37 °C in 95% air/5% CO_2 humidified atmosphere.

Expression of the main metabolic enzymes of 2-AG in human cytotrophoblasts and BeWo cells

Human cytotrophoblasts and BeWo cells were scraped in RIPA buffer and centrifuged at 14 000 ***g*** for 10 min at 4 °C. Proteins (100 μg) were subjected to 10% SDS–PAGE and transferred onto nitrocellulose membranes. After blocking non-specific binding sites with blocking buffer (5% of dry milk in PBS with Triton X-1000, 1%), the membranes were incubated with anti-MAGL and anti-DAGLA antibodies (1:100) overnight at 4 °C. Then, the membranes were incubated for 1 h, at room temperature, with peroxidase-conjugated secondary antibody (1:1000). Finally, the blots were analysed using a chemiluminescence detection kit and exposed to X-ray films. The membranes were then stripped and reincubated with anti-β-tubulin (1:500) for loading control. A negative control assay was conducted in one strip membrane where the primary antibody was omitted and only the secondary antibody was added to detect non-specific binding (data not shown). Rat brain was used as a positive control.

For immunocytochemistry, human cytotrophoblasts and BeWo cells (3×10^4 cells/well) were seeded in eight-well chamber slides and fixed with methanol, and the expression of DAGLA and MAGL was analysed using an avidin–biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ABC Kit). The non-specific binding sites were blocked and the slides were incubated with anti-DAGLA (1:100) or anti-MAGL (1:100) antibodies overnight at 4 °C. The cells were then incubated with biotinylated secondary antibody, followed by incubation with the Vectastain ABC-AP reagent, according to the manufacturer's instructions. The reaction was developed with Sigma Fast Red tablets. The slides were counterstained with Mayer's haematoxylin solution and mounted in Aquamount medium. Negative control assays were conducted in the absence of primary antibodies, which were replaced with rabbit IgG. Rat brain was used as a positive control.

Gene transcription of *MAGL* and *DAGLA* was evaluated by RT-PCR. Human cytotrophoblasts and BeWo cells were collected in TRIzol reagent and total RNA was extracted according to the manufacturer's instructions and quantified in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). RNA quality was assessed using the Experion RNA StdSens Kit, and the results were analysed with the Experion analytical software (Bio-Rad Laboratories). cDNA was obtained by RT of RNA using the iScript Select cDNA Synthesis Kit. For quantitative PCR, cDNA was amplified using the KAPA SYBR FAST qPCR Master Mix 2× Kit according to the kit protocol in the MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories). Primer sequences and PCR conditions are summarized in Table 1. The specificity of the amplified PCR products was assessed by the analysis of the melting curve.

Cell viability

BeWo cells were plated in 96-well plates at a density of 1×10^4 cells/well. After 24 h, the medium was replaced with DMEM/F12 medium with 1% FBS and 1% antibiotic solution in the presence or absence of 2-AG (0.01–25 μM), and the cells were incubated for 24, 48 and 72 h. The yellow tetrazole MTT (final concentration: 0.5 mg/ml) was added, and the cells were incubated for 2 h 30 min at 37 °C. The formed purple formazan was dissolved in a solution of DMSO:isopropanol (3:1) and spectrophotometrically quantified at 540 nm using a Multiskan Ascent microplate reader. The release of cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium was evaluated using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit according to the manufacturer's instructions.

Incorporation of ^{3}H-thymidine

Proliferative assays were carried out by the quantification of ^{3}H-thymidine incorporated in BeWo cells treated with increasing concentrations of 2-AG. ^{3}H-thymidine (final concentration: 0.5 μCi) was added 8 h before the end of the experiment. After two cycles of freezing/defrosting, the cells were harvested using a semi-automated cell harvester (Skatron Instruments, Lier, Norway), 1 ml of scintillation cocktail was added and ^{3}H-thymidine that was incorporated was quantified using a scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA, USA).

Table 1 RT-PCR conditions used to assess the gene expression of diacylglycerol lipase A (*DAGLA*) and monoacylglycerol lipase (*MAGL*) in BeWo cells.

Gene	GenBank	Primer sequence (5′–3′)	Annealing temperature (°C)	Amplicon length (bp)	Melting temperature (°C)	Reference
DAGLA	NM_006133.2	Forward: TGCTCTTCGGCCTGGTCTAT Reverse: CGCATGCTCAGCCAGATGAT	61	130	85.6	Ludanyi *et al.* (2008)
MAGL	BC000551.2	Forward: CAAGGCCCTCATCTTTGTGT Reverse: ACGTGGAAGTCAGACACTAC	57	162	85.5	Ludanyi *et al.* (2008)

Morphological studies

2-AG-induced morphological alterations in BeWo cells were evaluated by phase-contrast microscopy and Giemsa and Höechst staining. The cells (3×10^4) were seeded in eight-well chamber slides, treated with 2-AG (10 μM) for 48 h and fixed with methanol for Giemsa staining or 4% paraformaldehyde solution for Höechst staining. The cells were incubated with Giemsa stain for 30 min and analysed under a light microscope or exposed to 0.5 mg/ml Höechst 33342 (in PBS) for 20 min and observed under a fluorescence microscope equipped with an excitation filter with maximum transmission at 360/40 nm (Eclipse E400, Nikon, Japan).

Determination of caspase 3/7 and 9 activities

The cells (5×10^3) were seeded in a 96-well white plate and treated with 2-AG (10 μM) for 36 h, and at the end of the incubation time, Caspase-Glo 3/7 and Caspase-Glo 9 reagents were added to the cells according to the manufacturer's instructions. The plate was incubated at room temperature and the resultant luminescence was measured in relative light luminescence units (RLU) using the 96-well Microplate Luminometer (BioTek Instruments, Winooski, VT, USA). A negative control assay was conducted by co-incubation of 2-AG with a specific caspase inhibitor, Z-VAD-FMK, and a positive control assay was conducted using STS (100 nM), which was added 12 h before the end of the experiment. The results are expressed in RLU.

Assessment of mitochondrial membrane potential

BeWo cells were seeded in a 96-well black plate (density 1×10^4) and treated with 10 μM of 2-AG for 36 h. The cells were then washed with a PBS/sucrose 100 mM/protease inhibitor (PI) cocktail and incubated with a 100 nM $DiOC_6$ solution for 20 min at 37 °C in the dark. The $DiOC_6$ solution was removed to eliminate the background fluorescence, a PBS/sucrose/PI solution was added to the cells and the fluorescence was read using the Microplate Fluorimeter (BioTek Instruments Winooski, VT, USA) (excitation: 488 nm; emission: 525 nm). For the positive control, the cells were incubated with the mitochondrial membrane-depolarizing agent CCCP (10 μM) for 15 min, at 37 °C, before incubation with $DiOC_6$. The results are expressed as a percentage, comparing the loss of mitochondrial membrane potential of the 2-AG-treated cells with the untreated cells.

Measurement of intracellular reactive oxygen and nitrogen species

For the quantification of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) generated in BeWo cells after a 48-h treatment with 2-AG, cells seeded in a black 96-well plate were incubated with the probe DCDHF-DA for 1 h at room temperature. Fluorescence was measured using the Microplate Fluorimeter (BioTek Instruments) (excitation: 485 ± 10 nm; emission: 530 ± 12.5 nm). H_2O_2 (200 μM) was used as a positive control. The results are expressed as a percentage, comparing the increase in ROS/RNS production induced by 2-AG with the production in the untreated cells.

Signalling pathways involved in 2-AG-induced cell death

To understand the cellular mechanisms underlying the loss of cell viability and mitochondrial membrane potential, production of ROS/RNS and increase in caspase 3/7 and 9 activities, BeWo cells were pre-exposed to the following compounds: AM251 and AM630 (1 μM), antagonists of CB1 and CB2 receptors respectively; MCD (400 μM), a membrane cholesterol-depleting agent; AM404 (1 μM), an inhibitor of the putative membrane transporter of eCBs; and NAC (500 μM), an antioxidant. For these studies, the cells were pre-treated with CB antagonists and AM404 for 30 min, MCD for 1 h 30 min and NAC was co-incubated with 2-AG. AM251, AM630 and AM404 were dissolved in ethanol; MCD and NAC were dissolved in distilled water. Equimolar concentrations of the vehicles did not induce significant effects on the evaluated parameters (data not shown).

Statistical analysis

Statistical analysis was carried out using one- or two-way ANOVA, followed by the Bonferroni *post hoc* test to make pairwise comparisons of individual means when significance was indicated (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). The results are the mean of at least three independent experiments carried out in triplicate. Data are expressed as the mean±S.E.M., and differences were considered to be statistically significant at $P<0.05$.

Results

Expression of the main enzymes involved in 2-AG biosynthesis and degradation in human cytotrophoblasts and in BeWo cells

To investigate whether human cytotrophoblast cells possess the enzymatic machinery for 2-AG metabolism, we sought to identify the two key enzymes involved in the biosynthesis and degradation of this eCB, DAGLA and MAGL respectively. Western blot analysis revealed that these enzymes are expressed in primary human cytotrophoblasts (Fig. 1A). Gene transcription of *MAGL* and *DAGLA* was investigated by RT-PCR (Fig. 1B). The melting curves indicated that a specific PCR product was amplified in the reactions. Immunocytochemistry revealed a cytosolic expression for both the enzymes (Fig. 1C).

Since we intended to study the role of 2-AG in cytotrophoblast cell proliferation and apoptosis, we used a cytotrophoblast cell model, due to the spontaneous *in vitro* differentiation and fusion of human cytotrophoblasts. In this way, we also concluded that the mRNA and protein of both the enzymes are expressed in BeWo cells (Fig. 2A, B and C).

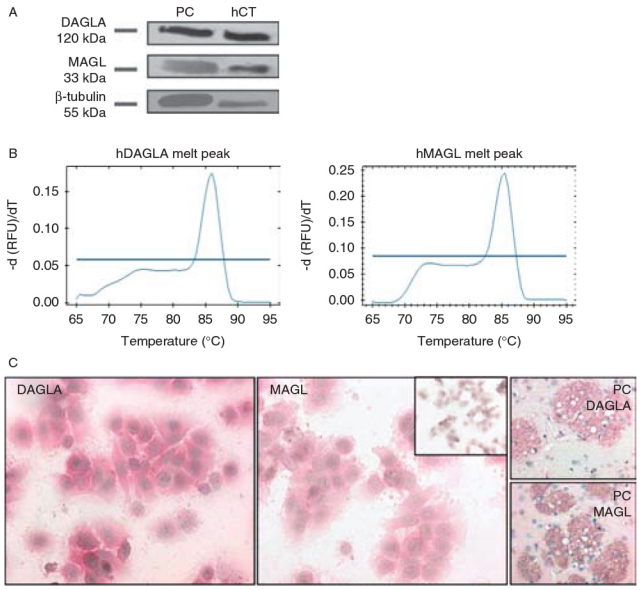

Figure 1 Expression of the main enzymes involved in 2-AG metabolism in human cytotrophoblasts (hCTs). (A) Western blot (WB) analysis revealed that both proteins are expressed in cytotrophoblast cells; β-tubulin was used as a loading control. (B) RT-PCR revealed that both genes are transcribed, as demonstrated by the specific PCR products represented in the melting curves. (C) Immunocytochemistry (ICC) revealed positive staining for diacylglycerol lipase A (DAGLA) and monoacylglycerol lipase (MAGL). Cells incubated without primary antibody were immunonegative (original magnification ×400). Rat brain homogenate and histological sections were used as positive control (PC) for WB and ICC respectively. 2-AG, 2-arachidonoylglycerol.

Effects of 2-AG on BeWo cell viability and proliferation

The treatment of BeWo cells with 2-AG (10–25 μM) induced a decrease in cell viability in a time- and dose-dependent manner (Fig. 3A). After 24 h of treatment, only the highest concentrations of 2-AG (25 μM) caused a significant reduction in cell viability. After 48 and 72 h, a remarkable decrease in cell viability was observed after treatment with 10–25 μM of 2-AG. It was observed that only 25 μM or higher concentrations of 2-AG induced the release of LDH (Fig. 3B).

The impact of 2-AG on cell proliferation was studied using the ^{3}H-thymidine incorporation assay, after 24 and 48 h of treatment (Fig. 3C), though a significant reduction in DNA synthesis with 10 μM or higher concentrations of 2-AG was observed only after 48 h.

Morphological alterations induced by 2-AG

The morphological alterations induced by 2-AG in BeWo cells were observed by Giemsa and Höechst staining. Cells treated with 2-AG (10 μM) for 48 h exhibited marked morphological alterations, such as the condensation and fragmentation of chromatin and the presence of apoptotic bodies (Fig. 4), typical features of apoptotic cell death.

Involvement of CB receptors, membrane cholesterol depletion and eCB membrane transporter in 2-AG-induced cell viability loss

To understand how 2-AG triggers the decrease in cell viability and morphological alterations in BeWo cells, the cellular signalling pathways that may be involved in these effects were investigated. It was observed that AM630, a selective antagonist of CB2 receptor, partially reversed the decrease in BeWo cell viability, whereas pre-treatment with the selective antagonist of CB1 receptor, AM251, had no effects (Fig. 5A). The eCBs can enter cells either by the putative eCB transporter or by simple diffusion. To investigate which mechanisms are involved in 2-AG signalling, BeWo cells were pre-incubated with AM404, an inhibitor of eCB transporter, or with MCD to deplete membrane cholesterol. Neither the blockage of the putative eCB transporter nor the depletion of cholesterol altered the effect on cell viability induced by 2-AG (10 μM) (Fig. 5B).

Induction of apoptosis in BeWo cells by 2-AG via a CB receptor- and mitochondria-dependent mechanism

As 2-AG-treated cells presented apoptotic features, to further investigate whether the viability loss effects were due to cell death via an apoptotic mechanism, caspase 3/7 and 9 activities and mitochondrial membrane

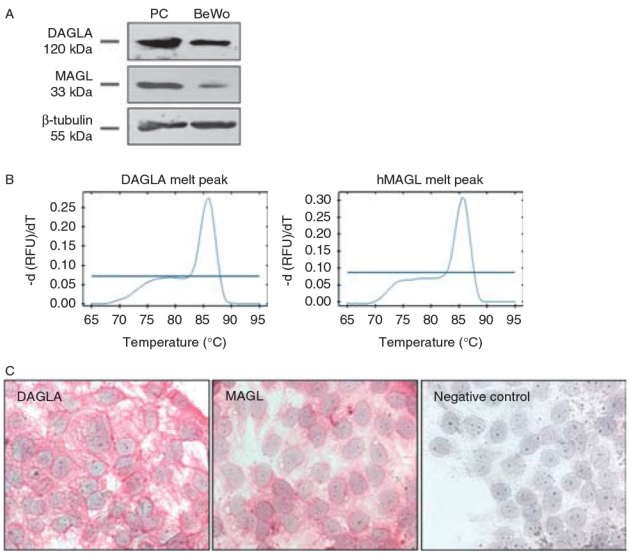

Figure 2 Expression of the main enzymes involved in 2-AG metabolism in BeWo cells. (A) Western blot analysis revealed that both proteins are expressed in BeWo cells; rat brain homogenate was used as positive control (PC) and β-tubulin was used as a loading control. (B) RT-PCR revealed that both genes are transcribed, as demonstrated by the specific PCR products revealed in the melting curves. (C) Immunocytochemistry revealed positive staining for diacylglycerol lipase A (DAGLA) and monoacylglycerol lipase (MAGL); cells incubated without primary antibody were immunonegative (original magnification ×400). 2-AG, 2-arachidonoylglycerol.

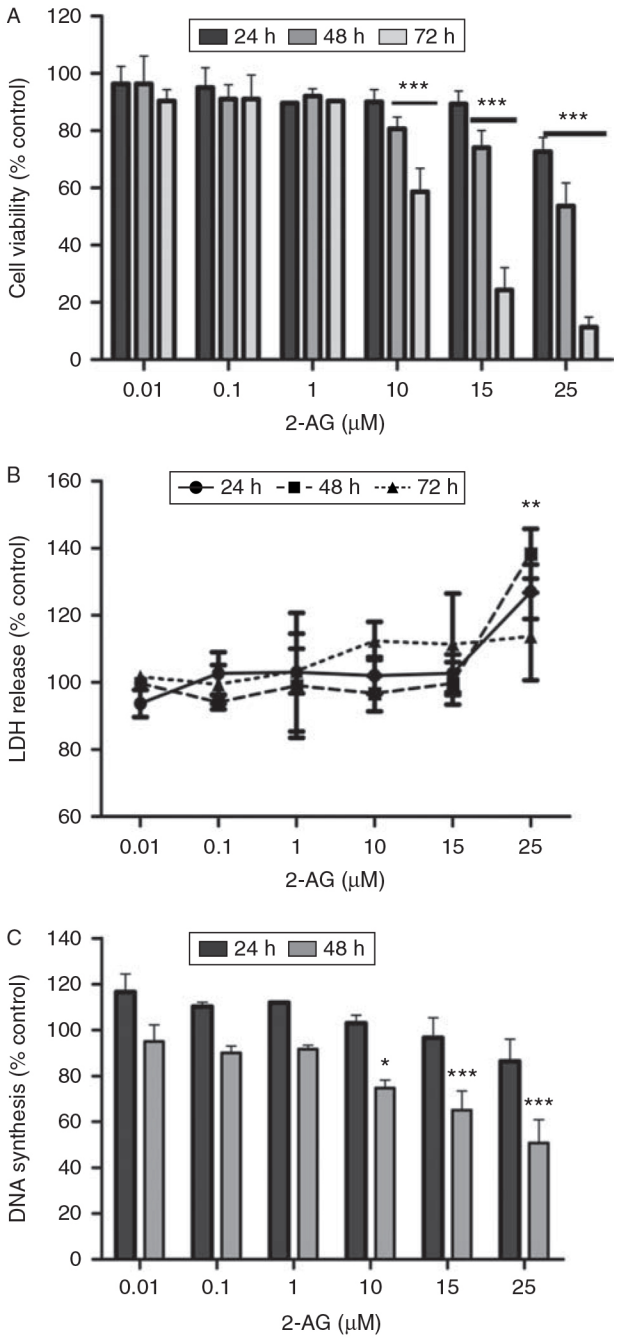

Figure 3 Effects of 2-AG on BeWo cell viability and proliferation, at different times of exposure (24, 48 and 72 h). (A) MTT assay revealed a reduction in cell viability in a time- and concentration-dependent manner (at concentrations higher than 1 μM). This reduction was accompanied by an increase in LDH release at 25 μM (B). (C) ^{3}H-thymidine incorporation decreased, after a 48-h treatment, with 10 μM of 2-AG (***$P<0.001$, **$P<0.01$ and *$P<0.05$ vs control). 2-AG, 2-arachidonoylglycerol; MTT, methylthiazolyldiphenyl-tetrazolium bromide; LDH, lactate dehydrogenase.

potential (Δψm) were evaluated. As caspase activation and Δψm loss precede cell viability loss, these parameters were evaluated after a shorter incubation time (36 h). As shown in Fig. 6A, 2-AG induced a Δψm loss of 23%, compared with that in the control cells, and the antagonists of both the CB receptors reversed this loss. The mitochondrial membrane-depolarizing agent CCCP induced a Δψm loss of 30%. An increase of 20 and 22% in caspase 3/7 and 9 activities respectively was observed in BeWo cells treated with 2-AG (10 μM) for 36 h, in comparison with the control cells (Fig. 6B and C). The observed increase in caspase 3/7 and 9 activities was significantly reversed only by AM630, antagonist of CB2 receptor.

Induction of the generation of intracellular ROS/RNS by 2-AG

The generation of ROS and RNS is implicated in several cellular events, including apoptosis. The ability of 2-AG to induce the production of ROS and RNS was assessed by fluorometry with the probe DCDHF-DA. A dramatic increase in the cellular generation of these species, relative to the control cells, was observed. Furthermore, it was verified that the production of ROS and RNS was CB receptor dependent, as both the antagonists used significantly prevented their generation (Fig. 7A). However, the antioxidant NAC failed to reverse the effects of 2-AG on cell viability (Fig. 7B).

Discussion

The study of endogenous cannabinoids revealed that cannabinoid signalling plays an important role in several pregnancy events such as embryo implantation, decidualization and labour. Thus, threats to ECS

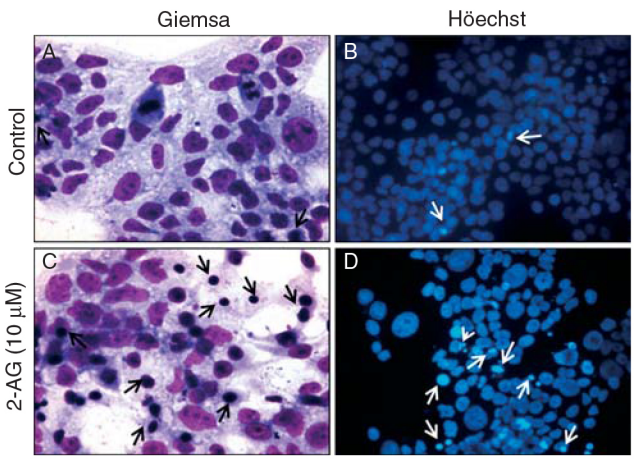

Figure 4 Morphological alterations induced by a 48-h treatment with 2-AG at a concentration of 10 μM in BeWo cells. Giemsa staining revealed cell shrinkage and an increase in the number of cells presenting chromatin condensation (arrows) (C) compared with the untreated cells (A). Höechst staining revealed the presence of chromatin condensation and fragmentation and the presence of apoptotic bodies (arrow head) in the treated cells (D), relative to the control cells (B). Original magnification ×400 (A and C) and ×200 (B and D). 2-AG, 2-arachidonoylglycerol.

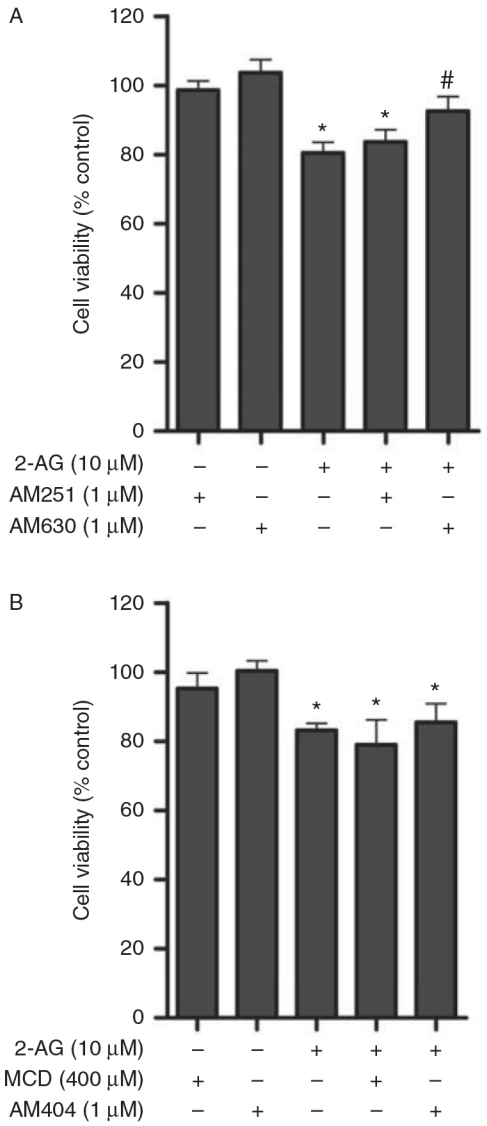

Figure 5 Effects of a 48-h treatment with 2-AG (10 μM) in BeWo cells pre-exposed to CB1 antagonist (AM251) and CB2 antagonist (AM630), eCB transporter inhibitor and MCD. (A) The CB2 antagonist partially reversed the decrease in cell viability induced by 2-AG. AM251 did not exert any effect (A). (B) The blockage of the putative eCB transporter by AM404 and the depletion of membrane cholesterol by MCD did not alter cell viability (*$P<0.05$ vs control; $^{\#}P<0.05$ vs 2-AG 10 μM). 2-AG, 2-arachidonoylglycerol; MCD, methyl-β-cyclodextrin.

homoeostasis are related to infertility and miscarriages (Paria *et al*. 1996, Wang *et al*. 2003, Schuel 2006, Fonseca *et al*. 2010, Taylor *et al*. 2010). The metabolic enzymes of AEA (NAPE-PLD and FAAH) have already been characterized in human placenta (Kenney *et al*. 1999, Park *et al*. 2003, Helliwell *et al*. 2004, Habayeb *et al*. 2008, Trabucco *et al*. 2009, Taylor *et al*. 2011), and it has also been reported that AEA induces a decrease in BeWo cell viability through the activation of CB2 receptor (Habayeb *et al*. 2008). On the other hand, only a few studies have examined a possible role for

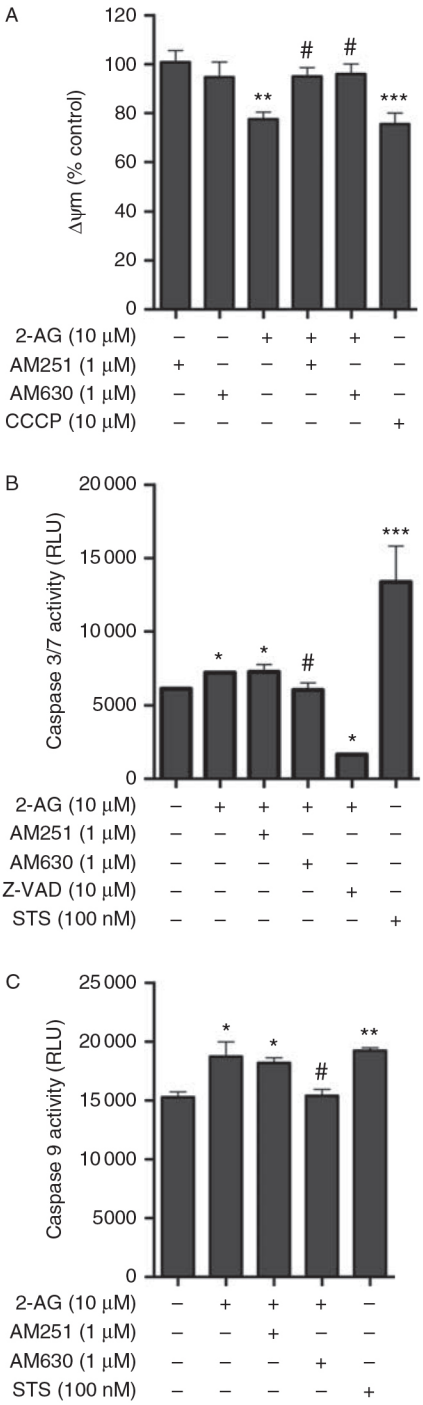

Figure 6 Evaluation of apoptotic markers in BeWo cells treated with 2-AG (10 μM) for 36 h. 2-AG induced a loss of mitochondrial membrane potential (Δψm), which was reversed by the antagonists of both CB1 and CB2 receptors (A). 2-AG induced an increase in caspase 3/7 (B) and 9 (C) activities, compared with that in the untreated cells; this increase was reversed by the CB2 antagonist AM630. STS (100 nM) and Z-VAD-FMK (10 μM) were used as positive and negative controls respectively for caspase 3/7 assay (***$P<0.001$, **$P<0.01$ and *$P<0.05$ vs control; $^{\#}P<0.05$ vs 2-AG 10 μM). 2-AG, 2-arachidonoylglycerol; DiOC6, 3,3′-dihexyloxacarbocyanine iodide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; STS, staurosporine.

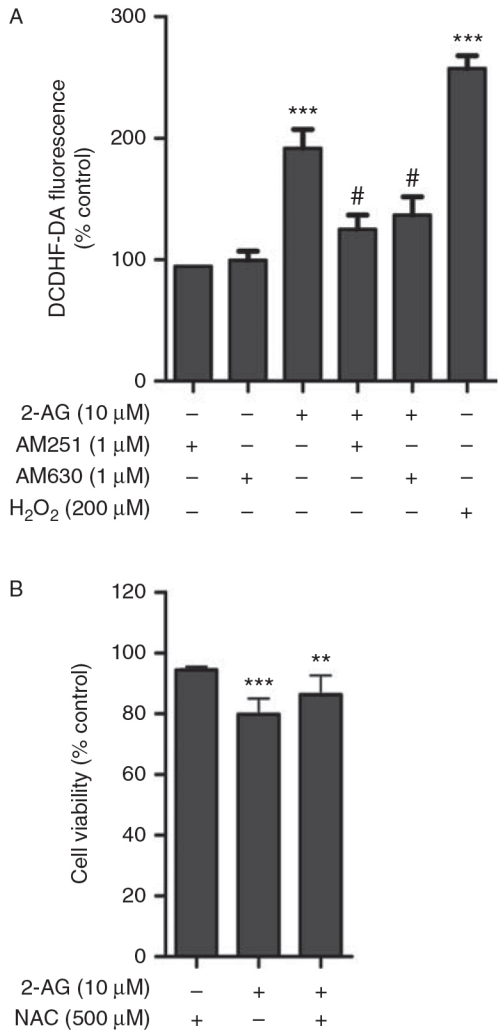

Figure 7 Modulation of ROS/RNS production by 10 μM of 2-AG after 48 h of treatment. 2-AG doubled the generation of ROS/RNS, with this effect being considerably attenuated by the antagonists of both the cannabinoid receptors (A). (B) The antioxidant NAC did not exert any effect on 2-AG-induced decrease in cell viability, suggesting that ROS/RNS generation is not directly involved in cell viability loss (***$P<0.001$ and **$P<0.01$ vs control; $^{\#}P<0.05$ vs 2-AG 10 μM). 2-AG, 2-arachidonoylglycerol; DCDHF-DA, 2,7-dichlorodihydrofluorescein diacetate; NAC, *N*-acetylcysteine.

2-AG in pregnancy. The levels of 2-AG in inter-implantation sites of mouse uterus (Wang *et al*. 2007) and in rat decidual tissue (Fonseca *et al*. 2010) are respectively 200- and 150-fold higher than those of AEA, suggesting that this eCB may be relevant to the regulation of implantation and uterine remodelling. In addition, it is known that 2-AG arrests mouse embryo development via CB1 receptor (Paria *et al*. 1998) and that the metabolic enzymes of 2-AG in mouse uterus regulate 2-AG during implantation (Wang *et al*. 2007). In this work, we demonstrate that human cytotrophoblasts and BeWo cells possess the enzymatic tools that regulate 2-AG levels *in situ*, suggesting that this eCB may be important for cytotrophoblast cell physiology. To our knowledge, this is the first time that 2-AG metabolic machinery is characterized in trophoblast cells. Moreover, the most widely used trophoblast-like cell model, BeWo cells, also expresses these key enzymes of 2-AG metabolism. For this reason and as human cytotrophoblasts spontaneously differentiate to form syncytiotrophoblasts in primary cell culture, not allowing the study of cytotrophoblast cell turnover, BeWo cells were chosen to investigate the effect of 2-AG on cytotrophoblast cell turnover.

The development of placenta is a highly coordinated and regulated event where trophoblast cells proliferate, differentiate and undergo apoptosis. It is well known that anomalies in these cellular processes impair placentation and are implicated in some pregnancy pathologies, such as pre-eclampsia and IUGR (Huppertz *et al*. 2006). Our results revealed that 2-AG induces a decrease in BeWo cell viability (at concentrations higher than 1 μM) and also exerts antiproliferative effects, in both a time- and a concentration-dependent manner. The inhibitory action of 2-AG on cell proliferation has already been reported in rat C6 glioma cells (Jacobsson *et al*. 2001) and colorectal carcinoma cells (Ligresti *et al*. 2003), both the situations being mediated by CB receptors. Herein, we report that the antiproliferative effects are accompanied by the presence of morphological alterations such as the condensation and fragmentation of chromatin and the presence of apoptotic bodies, typical features of cell death by apoptosis. Moreover, this eCB induced a loss of mitochondrial membrane potential, an increase in caspase 3/7 and 9 activities and an increase in ROS/RNS production. However, whereas the increase in caspase 3/7 and 9 activities and the loss of cell viability were reversed by the antagonist of CB2 receptor, the other studied parameters were reversed by the antagonists of both the CB receptors. These data suggest that the mitochondrial pathway may be involved in 2-AG-induced apoptosis and that this mechanism is CB receptor dependent. This is in agreement with the data obtained with the inhibition of eCB transporter and alterations in membrane integrity that did not reverse the effects of 2-AG. The inability of AM251 to reverse the increase in caspase 3/7 and 9 activities and loss of cell viability is not totally clarified, and further studies have to be carried out to elucidate the underlying mechanisms that result from the activation of this receptor. However, CB1 receptor is important for the loss of mitochondrial membrane potential and the generation of ROS/RNS, events that precede cell viability loss and may also occur before caspase activation. On the other hand, the activation of CB2 receptor seems to be crucial for the mitochondria-dependent apoptotic stimulus triggered by 2-AG in BeWo cells. Although the pro-apoptotic effects of 2-AG have already been described in other cell types, the mechanism may be cell specific. Indeed,

2-AG induces apoptosis in rat decidual cells by the activation of CB1 receptor (Fonseca *et al*. 2010) and in hepatic stellate cells through the generation of mitochondrial ROS (Siegmund *et al*. 2007). Additionally, it has been reported that the other 'major' eCB, AEA, induces a decrease in BeWo cell viability through a mechanism involving CB2 receptor, although the pathways behind this effect have not been totally clarified (Habayeb *et al*. 2008). These data reinforce a role for the ECS in cytotrophoblast cell turnover and the relevance of the signalling pathways initiated by the activation of CB2 receptor during this process. Besides, 2-AG may be hydrolysed into arachidonic acid, the source of prostaglandins, by MAGL, or metabolized by oxidative enzymes (Fonseca *et al*. 2013). In addition, as BeWo cells express cyclooxygenase 1 (COX1) and 2 (COX2) (Xu *et al*. 2005), prostaglandins and 2-AG oxygenated metabolites may be involved in 2-AG-mediated cell death, as CB antagonists only partially reverse these effects. Prostaglandins can induce cell death in several cell types (Pignatelli *et al*. 2005, Huang *et al*. 2009, Liu *et al*. 2013), and it is known that the COX2 metabolites of 2-AG, the prostaglandin glyceryl esters, possess biological activity (Nirodi *et al*. 2004, Sang *et al*. 2007). Additionally, the COX2 metabolites of AEA, the prostamides, can induce cell death in some cell types (Patsos *et al*. 2010, Kuc *et al*. 2012). Further investigation is required to clarify the role of these lipid mediators in cytotrophoblast cell turnover.

During placentation, trophoblasts are in continuous turnover and apoptosis is a key event for the normal formation of the organ, with both extrinsic and intrinsic apoptotic pathways being involved in this cellular event (Heazell & Crocker 2008). Moreover, members of the Bcl-2 family are differently expressed during the gestational period in cytotrophoblasts and syncytiotrophoblasts (Ishihara *et al*. 2000, De Falco *et al*. 2001), with alterations in their expression being described in some pathophysiological situations such as pre-eclampsia, IUGR and hyperglycaemia (Sgarbosa *et al*. 2006, Tomas *et al*. 2011, Longtine *et al*. 2012*b*, Borzsonyi *et al*. 2013). Therefore, it seems that anomalies in cell death regulation through the mitochondrial pathway of apoptosis may be involved in pregnancy complications. Also, villous cytotrophoblasts undergo caspase-dependent apoptosis and apoptosis rates in these cells are increased in complicated gestations (Longtine *et al*. 2012*a*, 2012*b*).

Pregnancy is a natural condition of oxidative and nitrative stress. However, greater ROS and/or RNS generation and decreased placental antioxidant defences highly increase the carbonylation and nitration of important proteins and the peroxidation of lipids, altering the biological function of these molecules (Myatt 2010, Burton & Jauniaux 2011). Moreover, these changes in placental redox state have been reported to be related to some pregnancy complications (Coughlan *et al*. 2004, Sharma *et al*. 2006, Biri *et al*. 2007), and it has been reported that BeWo cell turnover is affected by oxidative stress (Heazell *et al*. 2009). In this way, considering that 2-AG induces a dramatic increase in the production of ROS and RNS in BeWo cells, the deregulation of cannabinoid signalling may be implicated in alterations of the oxidative state of cytotrophoblasts, interfering with the normal course of gestation.

In conclusion, the existence of the two key enzymes involved in 2-AG biosynthesis and degradation, DAGLA and MAGL, in cytotrophoblast cells allows proper regulation of 2-AG levels *in situ*. Moreover, the antiproliferative effects and the induction of apoptosis implicate 2-AG in cytotrophoblast cell turnover through the activation of CB2 receptor. Therefore, this eCB may be an important intervener in the development of placenta. Thus, this work highlights the relevance of eCB signalling in placenta, not neglecting that deregulation in ECS homoeostasis may have deleterious effects on cytotrophoblast cell turnover and contribute to the pathophysiological mechanisms of some pregnancy complications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

The authors thank Fundação para a Ciência e Tecnologia (FCT) for the PhD grant of Mariana Costa (SFRH/BD/70721/2010) and for the post-doctoral grant of Bruno Fonseca (SFRH/BPD/72958/2010) and Obstetrics and Gynaecology Service of Hospital S. João.

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Received 4 November 2013
First decision 25 November 2013
Revised manuscript received 29 November 2013
Accepted 9 December 2013

Manuscript II

The endocannabinoid anandamide induces apoptosis in cytotrophoblast cells: involvement of both mitochondrial and death receptor pathways.

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Placenta, 2014. DOI: 10.1016/j.placenta.2014.10.011

Contents lists available at ScienceDirect

Placenta

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The endocannabinoid anandamide induces apoptosis in cytotrophoblast cells: involvement of both mitochondrial and death receptor pathways

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Key words:
Anandamide; apoptosis; cytotrophoblasts; endocannabinoid signalling; placenta

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ABSTRACT

Introduction: A balanced proliferation, apoptosis and differentiation in trophoblast cells of the human placenta is crucial for a proper placental development. Alteration in trophoblast apoptosis and differentiation are associated with gestational-related complications, such as preeclampsia, intrauterine growth restriction or miscarriages. The endocannabinoids (eCBs) have been recognized as new interveners in pregnancy events such as implantation and decidualization. However, their importance in placentation is poorly understood. We hypothesise that these novel lipid mediators may intervene in cytotrophoblast apoptosis and, concomitantly, have a role during placental development.

Methods: primary human cytotrophoblasts (hCTs) and the human trophoblast-like choriocarcinoma cell line BeWo cells were exposed to Anandamide (AEA). It was investigated the cellular pathways involved in cell death, by the assessment of cell morphology, caspases activity, mitochondrial membrane potential ($\Delta\psi m$), reactive oxygen/nitrogen species (ROS/RNS) and western blot of cleaved Poly (ADP-ribose) polymerase 1 (PARP-1), truncated Bid (t-Bid) and IκB-α.

Results: AEA decreased hCTs viability and induced morphological features of apoptosis (chromatin condensation and fragmentation), caspase-3/7 activation and PARP-1 cleavage. In BeWo, AEA also increased the activities of caspase-3/7 and 9, induced loss in $\Delta\psi m$ and production of ROS/RNS. These effects were reversed by either CB1 or CB2 antagonists, whereas the increase in caspase 3/7 activity was only reversed with CB2 blockage. AEA-treated cells showed increased caspase-8 activation and formation of t-Bid, suggesting the interplay between intrinsic and extrinsic apoptotic pathways. AEA also increased IκB-α expression, a NF-κB regulatory protein.

Conclusion: Our results highlight the importance of eCBs in cytotrophoblast cell apoptosis and indicate that a crosstalk between intrinsic and extrinsic apoptotic pathways is involved in AEA-induced effects.

1. Introduction

The placenta is a specialized organ with vital functions, such as nutrient and gas exchange, immunomodulation and protein biosynthesis. The main placental cells, the trophoblasts, are subdivided in different cell types: cytotrophoblasts, syncytiotrophoblast and extravillous trophoblasts (EVTs). Cytotrophoblasts are mononuclear cells that proliferate, fuse and differentiate into other types of trophoblasts. The syncytiotrophoblast is a multinucleated layer that produces several hormones and proteins and is in direct contact with maternal blood, allowing the mother-foetus communication. The EVTs have invasive properties and are involved in the uterine blood vessels remodelling [1]. A coordinated proliferation, differentiation and apoptosis of trophoblasts is required for a proper placental development, and any disturbance in these processes is associated with gestational complications [2-7].

The Endocannabinoid System (ECS) has emerged as a key modulator of multiple physiological and pathophysiological processes, including reproductive events like implantation and decidualization. This system comprises the two cannabinoid receptors CB1 and CB2, their endogenous ligands (endocannabinoids- eCBs), the enzymes involved in eCBs biosynthesis and degradation and a putative membrane transporter (See [8] for review). Anandamide (AEA) or *N*-arachidonoylethanolamine was identified in 1992 in pig brain [9] and is currently the best studied eCB. It is synthesised from membrane phospholipids in a two-step reaction catalysed by the enzymes *N*-acyltransacylase and *N*-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD). After exerting its effect, it is hydrolysed inside the cell, mainly by Fatty Acid Amide Hydrolase (FAAH), an inner cellular membrane enzyme. This eCB induces apoptosis in several cell types by activating different receptors and cell signalling pathways. In fact, it was described that AEA-induced apoptosis involve the activation of its main targets, the CB receptors, triggering several downstream pathways, including activation of mitogen-activated protein kinases (MAPKs), increase of ceramide levels or intracellular Ca^{2+} and generation of oxidative stress [10-14].

The importance of cannabinoid signalling homeostasis was only been recently recognized. In fact, a proper AEA tone is required for embryo transport along the mouse oviduct [15] and low levels of this eCB are required in implantation sites and are mainly regulated locally by the enzyme FAAH (see [16] for review). Nevertheless, information about the role of AEA during the period of placental development is lacking. It was reported that CB receptors and AEA main metabolic enzymes are expressed in first trimester and term placentas [17-20]. Also, in preeclamptic placentas, NAPE-PLD expression was increased whereas FAAH was decreased in comparison to normal placentas, suggesting a function for eCBs in the pathophysiology of preeclampsia [21]. It was also described that AEA decreases the viability of BeWo cells, via CB2 receptor [20]. Moreover, we have recently demonstrated that the other major eCB, 2-arachidonoylglycerol (2-AG), has the ability to induce apoptosis in BeWo cells through a CB receptor- and mitochondrial-dependent mechanism [22].

Here, we investigate the impact of AEA on cytotrophoblasts viability and explore the cellular mechanisms triggered by this endocannabinoid. We hypothesise that this major endocannabinoid may integrate the network of hormones, cytokines and other molecules that regulate cytotrophoblast proliferation and apoptosis.

2. Materials and Methods

All chemicals were from Sigma-Aldrich Co. (St. Louis, MO, USA), except: Anandamide, AM251 and AM630 (Tocris Bioscience, Bristol, UK); 3, 3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Gibco/Invitrogen Corporation, Carlsbad, CA, USA); Z-VAD-fmk (BD PharMingen, San Diego, CA); CytoTox 96 nonradioactive cytotoxicity assay kit, Caspase-Glo® (Promega, Madison, WI, USA); ^{3}H-thymidine (Amersham, Aylesbury, UK); percoll (GE Healthcare, Buckinghamshire, UK); and WesternBright™ ECL HRP substrate (Advansta, Menlo Park, USA).

2.1 Isolation and culture of human cytotrophoblasts

All the procedures were performed in accordance with the Ethical Committee of Hospital S. João, Porto (authorization nº 237-13). For each assay, cytotrophoblasts were isolated from five different term placentas from clinically normal pregnancies, according to a modification of the Kliman's protocol, as previously described [23]. Briefly, decidual tissue was removed, villous tissue was collected from at least 10 different regions homogeneously distributed in the whole placenta, and the major blood vessels were discarded by fine dissection. Then, the tissue was digested in a trypsin and DNAse-containing solution and the obtained cells were separated in a discontinuous percoll gradient, at 1200 g. The cytotrophoblasts were collected and incubated at 37 °C and 95% air/ 5% CO_2 humidified atmosphere, in DMEM/F12 medium supplemented with 10% (v/v) FBS and antibiotic–antimycotic solution (100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericine B). To evaluate the purity of the cytotrophoblast cultures, cells were fixed with a 4% paraformaldehyde solution and immunostained with anti-cytokeratin-7 and anti-vimentin antibodies. Around 95% of the cells were cytokeratin-7 positive, which corresponds to the epithelial hCT cells. For the experiments, cells were plated in 6- and 96-well plates or 8-well chamber slides, at densities 4.5 x 10^5, 1.5 x 10^5 or 5 x 10^5 cells/well, respectively.

2.2 BeWo cell culture conditions

The human choriocarcinoma cell line BeWo (ATCC, Manassas, VA, USA) was incubated at 37 °C and 95% air/ 5% CO_2 humidified atmosphere, in DMEM/F12 medium supplemented with 10% (v/v) FBS and an antibiotic–antimycotic solution. For the experiments, cells were used between 78 and 90 passages and seeded in 96 or 6-well plates and 8-well chamber slides, at densities 1 x 10^4, 2 x 10^5 and 3 x 10^4 cells/well, respectively. At least five independent experiments performed in triplicate for each assay.

For the investigation of cellular pathways triggered by AEA, BeWo cells were pre-treated for 30 min with CB1 and CB2 antagonists, AM251 or AM630 (1 µM), respectively. AEA, AM251 and AM630 were dissolved in ethanol. Equimolar concentrations of the vehicle did not induce any effects on all the parameters (data not shown).

2.3 Cell viability and cytotoxicity assays

The hCTs and BeWo cells were plated in 96-well plates and, after adhesion, incubated in DMEM/F12 medium with 1% FBS and 1% antibiotic solution, in the absence or presence of AEA (1-25 µM) for 12 and 24 h (hCTs) or for 24, 48 and 72 h (BeWo cells). The yellow tetrazole MTT (0.5 mg/ml final concentration) was added and cells were incubated for 2 h 30 min, at 37 ºC. The resultant purple formazan was extracted by a solution Dimethylsulfoxide (DMSO):isopropanol (3:1) and quantified by spectrophotometry, at 540 nm, in a Multiscan Ascent microplate reader. The activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) released into the culture medium was assessed by the CytoTox 96 nonradioactive cytotoxicity assay kit, according to the manufacturer's instructions.

2.4 Morphological studies

The alterations in cell morphology induced by AEA (15 µM) treatment of hCTs for 24 h or AEA (10 µM) treatment of BeWo for 48 h were analysed by Giemsa and Höechst staining. These concentrations were selected according to the viability assays of each cellular model. After the treatment, cells plated in 8-well chamber slides were fixed with a 4% paraformaldehyde solution, stained with Giemsa and analysed under light microscopy. For Höechst staining, cells were exposed to 0. 5 mg/ml Höechst 33342 for 20 min and examined under a fluorescence microscope (Eclipse E400, Nikon, Japan) equipped with an excitation filter with maximum transmission at 360/40 nm.

2.5 Incorporation of 3H-thymidine

The impact of AEA in BeWo cells proliferation was evaluated by the quantification of ^{3}H-thymidine incorporation in the absence or presence of AEA (1-25 µM) for 24 and 48 h. ^{3}H-thymidine (0.5 µCi final concentration) was added to each well, 8 h before the end of the incubation time. Cells were frozen/thawed twice, harvested with a semi-automated cell harvester (Skatron Instruments, Lier, Norway). After the addition of scintillation cocktail, the incorporation of ^{3}H-thymidine was quantified in a scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA, USA).

2.6 Determination of Caspase-3/7, 8 and 9 activities

The activity of caspases was determined by luminescence assays by using caspase-Glo®-3/7, 8 or 9 assays, according to manufacture instructions. Cells were seeded in 96-well white plates and exposed to 15 μM of AEA for 20 h (in case of hCTs) or to 10 μM of AEA for 36 h (in case of BeWo cells). The plates were incubated for 1 h at room temperature and the resultant luminescence was measured in a Microplate Luminometer (BioTek Instruments, Winooski, VT, USA) and presented as in relative light units (RLU). A negative control was performed by co-incubation of AEA with a specific caspase inhibitor, Z-VAD-fmk (20 μM) and a positive control was performed with the apoptosis inductor staurosporine (STS; 100 nM) added 12 h before the end of experiment.

2.7 Evaluation of mitochondrial membrane potential (Δψm) and intracellular reactive oxygen and nitrogen species (ROS/RNS)

For the assessment of Δψm and ROS/RNS production, BeWo cells were seeded in 96-well black plates and treated with AEA (10 μM) for 36 h or 48 h, respectively. For Δψm studies, cells were washed and incubated with $DiOC_6$ 100 nM, for 20 min, at 37 ºC, in the dark. For the evaluation of ROS/RNS production, cells were washed and incubated with the fluorescent probe 2,7-Dichlorodihydrofluorescein diacetate (DCDHF-DA), for 1 h, at room temperature. For both assays, the resulting fluorescence was measured in a Microplate Fluorimeter (BioTek Instruments, Vermont, USA) (excitation-485+/-10 nm; emission 530+/-12, 5 nm). The positive controls for Δψm or ROS/RNS production were the mitochondrial depolarizing carbonyl cyanide m-chlorophenylhydrazone (CCCP; 10 μM) or H_2O_2 (200 μM), respectively. The results were expressed in relative fluorescence units (RFU).

2.8 Western Blot analysis

Western Blot was used for the assessment of cleaved PARP-1 in hCTs and Bid/t-Bid and IκB-α (Table 1). BeWo cells were seeded in 6 well plates and incubated in the absence or presence of AEA alone or pre-incubated with CB receptors antagonists. After 24 or 48 h, for hCTs and BeWo respectively, cells were lysed in ice-cold lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% Triton™ X-100). Proteins were resolved in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking nonspecific binding sites, membranes were incubated with the corresponding primary antibody, overnight, at 4 ºC, and then with peroxidase-conjugated secondary antibody for 1 h, at room temperature. Lastly, a Western Bright™ ECL was added to the membranes and exposed to x-ray film. Membranes were then stripped and reincubated with anti-tubulin antibody for loading control. The signal intensity was quantified by densitometry (BIO-PROFIL Bio-1D2; Vilber Lourmat, Marne-la-Vallée, France) and the results expressed in arbitrary

Table 1. Summary of the electrophoresis conditions. All the antibodies were from Santa Cruz Biotechnology (Dallas, Texas, USA), except anti-Bid, which was from Cell Signaling Technology (ZA Leiden, Netherlands)

Protein	Molecular Weight (kDa)	SDS-PAGE (%)	Positive control	Primary antibody		Secondary antibody	
				source	dilution	source	dilution
IκB-α	35	10 %	Hela cells extract	Rabbit	1:400	Goat anti-rabbit	1:2000
Cleaved PARP-1	85	10 %	BeWo cells treated with STS	Rabbit	1:100	Goat anti-rabbit	1:1000
BID/t-BID	22/15	15 %	Hela cells extract	Rabbit	1:200	Goat anti-rabbit	1:1000
β-tubulin	55			Rabbit	1:750	Goat anti-rabbit	1:3000

units, after normalization for the corresponding β-tubulin band.

2.9 Statistical analysis

Statistical analysis was carried out by Wilcoxon signed-rank, Mann-Whitney or Kruskal-Wallis tests followed by multiple comparisons with Dunn's test (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). The results are the mean of at least five independent experiments performed in triplicate. Data were expressed as mean ± SEM and differences were considered to be statistically significant at $p<0.05$.

3. Results

3.1 AEA effects in primary cultures of human cytotrophoblasts

Treatment of hCTs with AEA decreased cell viability, in a concentration dependent manner, for concentrations higher than 15 μM (Figure 1A). Similar effects were observed at 12 and 24 h of treatment. LDH release was also evaluated to study the cytotoxic effects of AEA. Only the highest concentration (25 μM) induced a significantly release of this enzyme (Figure 1B). The impact of AEA in hCTs morphology was investigated by Giemsa and Höechst staining. Cells treated with AEA (15 μM) showed chromatin condensation and fragmentation (Figures 1C-F). In addition, it was observed an increase of 28% in caspase-3/7 activity, after 20 h of treatment (Figure 1G) and an increase in PARP-1 cleavage (Figure 1H).

3.2 AEA effects in cell viability and proliferation of BeWo cells

The human cytotrophoblasts spontaneously differentiate *in vitro* into syncytiotrophoblasts, after 72 h of culture [24], hampering the study of the cytotrophoblast cell population in primary cultures. Thus, BeWo cells were chosen as a model to carry out the investigation of the cellular pathways triggered by AEA. These cells are widely used as a representative model of hCTs since they are able to proliferate and do not significantly differentiate without a stimuli, to allow the study of AEA effects on cytotrophoblast viability. Additionally, they express CB receptors and other members of ECS and respond to cannabinoid stimuli [20, 22]. The exposure of BeWo cells to AEA diminished cell viability, in a concentration and time dependent manner (Figure 2A). In fact, a 24 h treatment with 15 μM AEA significantly decreased cell viability by 26%. After a 48 h treatment, a cell viability loss was observed with 10 μM AEA (about 20%) and a dramatic reduction on cell viability was observed with 25 μM (by 74%). At 72 h of treatment, lower concentrations of AEA (5 μM) affected cell viability and 10 μM induced a massive cell death (around 55%). LDH release was assessed in order to evaluate the involvement of a cytotoxic mechanism in AEA effects on cell viability (Figure 2B). Higher concentrations than 25 or 15 μM significantly increased LDH release, after 24/48 h or 72 h of treatment, respectively. The ^{3}H-thymidine incorporation assay showed that BeWo cells proliferation was also decreased by 23% with 15 or 10 μM AEA, after a 24 or 48 h exposure, respectively (Figure 2C). This effect was more notorious with higher concentrations of AEA.

In the following experiments that aim to study the cellular pathways involved in AEA-induced cell death, BeWo cells were treated with 10 μM of AEA for 48 h, since in these conditions AEA decreased cell viability and proliferation without inducing LDH release. To clarify the involvement of CB receptors in the cellular mechanisms behind the AEA-induced decrease in BeWo cells viability, cells were pre-treated with selective antagonists of CB1 and CB2, AM251 and AM630, respectively, which *per se* did not interfere with cell viability (Figure 2D).

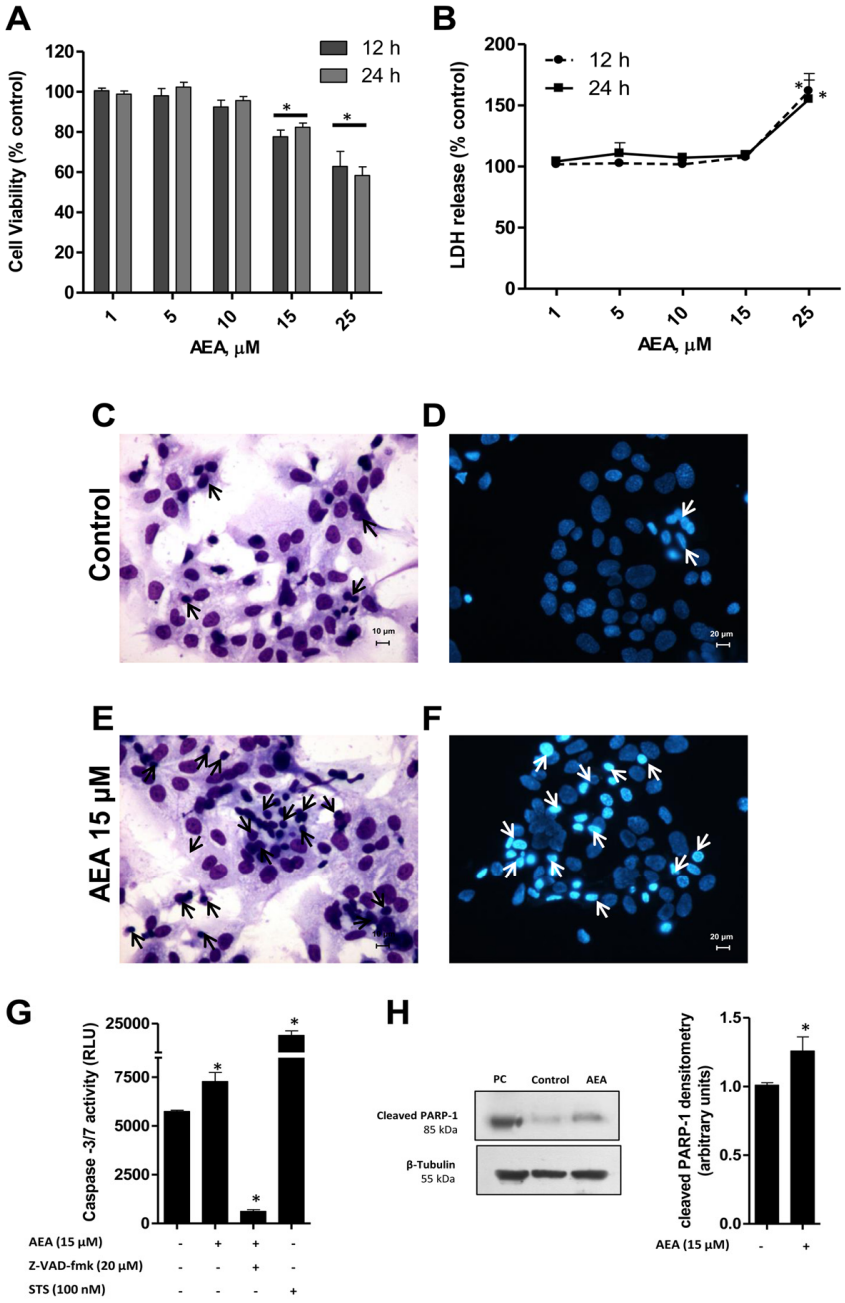

Figure 1. Anandamide (AEA) effects in human cytotrophoblast cells (hCTs). (A) MTT assay showed that AEA decreased hCTs viability after 12 and 24 h of treatment, in a concentration-dependent way, for concentrations higher than 15 µM and (B) LDH release was only significantly increased for 25 µM. Giemsa staining revealed that the 24 h-treatment with 15 µM of AEA induced chromatin condensation (arrows) (E), in comparison to the control (C). Höechst staining confirmed the presence of nuclei with chromatin condensation (arrows) in AEA-treated hCTs (F), in comparison with the control (D). (G) Caspase 3/7 activity was increased in cells treated with AEA for 20 h and (H) there is an increase of PARP-1 cleavage after a 24 h treatment; STS was used as positive control. (*p < 0.05 vs. Control; Wilcoxon signed-rank test for A and B; Mann-Whitney for G and H). LDH- Lactate dehydrogenase; MTT- Methylthiazolyldiphenyl-tetrazolium bromide; STS- Staurosporine.

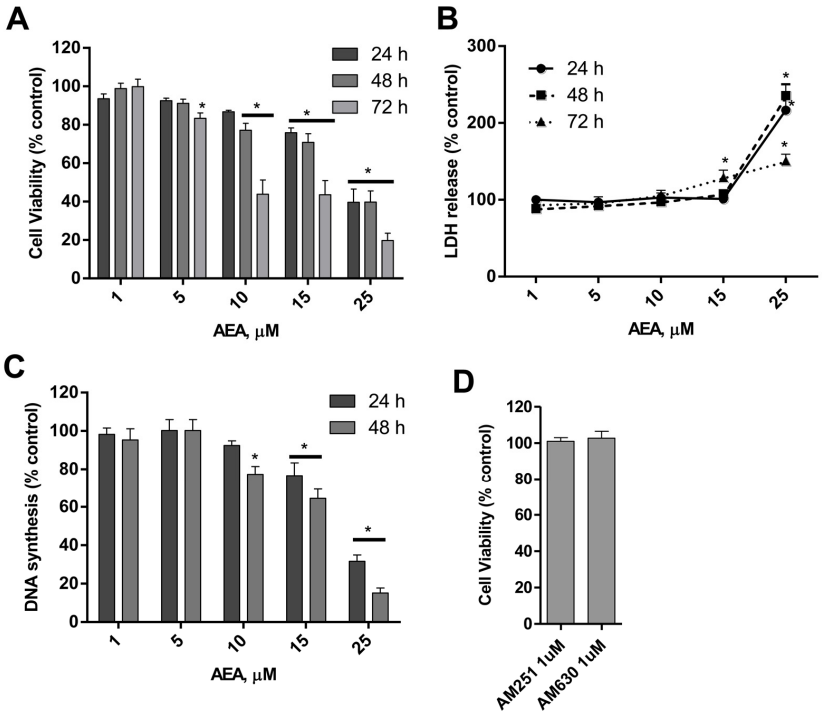

Figure 2. Anandamide (AEA) effects in BeWo cells viability and proliferation, after 24, 48 or 72 h of exposure. (A) MTT assay revealed a decrease in cell viability, in a concentration and time -dependent manner (for concentrations higher than 5 μM). (B) LDH release was significantly increased for concentrations higher than 20 μM, for 24 and 48 h treatments; and then 15 μM, for 72 h treatment. (C) The incorporation of ^{3}H-thymidine was decrease for 10 μM or higher concentrations of AEA. (D) CB receptors antagonists, AM251 and AM630 (1 μM), did not interfere with cell viability, after a 48 h-treatment. (*$p < 0.05$ vs. Control; Wilcoxon signed-rank test). MTT-Methylthiazolyldiphenyl-tetrazolium bromide; LDH-Lactate dehydrogenase.

3.3 AEA effects in BeWo cells morphology

The morphological alterations induced by AEA in BeWo cells were observed by Giemsa and Höechst staining. AEA-treated cells (10 μM) presented morphological features that are characteristic of apoptosis like chromatin condensation and fragmentation and the presence of apoptotic bodies (Figure 3).

3.4 Study of the mechanisms of cell death triggered by AEA in BeWo cells

As morphological studies indicated the presence of apoptotic features, we evaluated AEA effects on the activity of the effector caspases-3/7 and also the involvement of intrinsic pathway, by the assessment of caspase 9 activity and mitochondrial membrane potential ($\Delta\psi m$). We found that this eCB induced a 16% increase in caspases-3/7 activity, effect that was partially reversed by the CB2 antagonist (Figure 4A). Moreover, AEA caused an increment of 24% of caspase-9 activity (Figure 4B) and a loss of 16% of mitochondrial membrane potential and the effects were partially reversed by both CB receptors antagonists, AM251 and AM630 (Figure 4C). The effects of AEA in the production of ROS/RNS were also evaluated, using the probe DCDHF-DA. AEA dramatically exacerbated the generation of ROS/RNS, in comparison with control. Both CB receptors antagonists were able to partially reverse this effect (Figure 4D).

To explore if the extrinsic pathway was triggered by AEA in BeWo cells, we investigated the effects of this eCB in proteins related to the extrinsic pathway, caspase-8 activity and t-Bid, and also in IκB-α, a regulatory protein that inhibits the NF-κB transcription factor. It was detected an increase of 19% in the activity of caspase-8, through a mechanism involving both CB1 and CB2 receptors (Figure 5A) and an increase in the formation of t-Bid (Figure 5B). Moreover, AEA also increased the expression of IκB-α, suggesting that NF- κB is not activated (Figure 5C). AEA effects on t-Bid and IκB-α were attenuated by CB1 and CB2 antagonists, though without statistical significance.

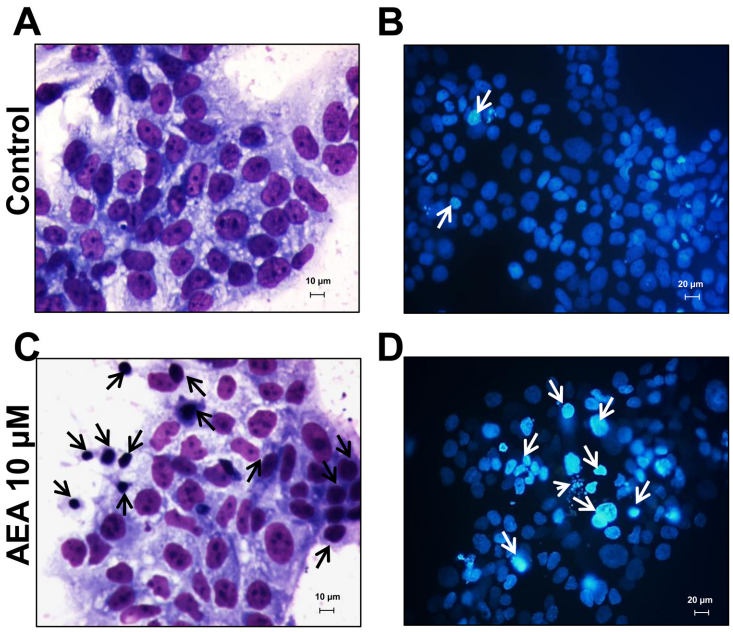

Figure 3. Morphological analysis of BeWo cells treated with anandamide (AEA) for 48 h. In Giemsa staining, cells treated with AEA (10 µM) presented cell shrinkage and chromatin condensation (arrows) (C) compared to untreated cells (A). Höechst assay also revealed cells with chromatin condensation (arrows) and the presence of apoptotic bodies (arrow head) in AEA-treated cells (D), in comparison with the control (B).

4. Discussion

The endocannabinoid signalling participates in the modulation of several physiological and pathophysiological events in different tissues and organs. AEA has been pointed out as a player in gestational events like implantation [25] and decidualization [26], where it induces apoptosis through CB1 receptor activation. However, the role of AEA in placental development is not completely understood. In this work, we report that AEA causes a decrease in cell viability of primary human cytotrophoblasts accompanied by apoptotic morphological features and biochemical markers such as caspase-3/7 activation and cleavage of PARP-1. Human cytotrophoblasts spontaneously differentiate *in vitro* into syncytiotrophoblasts, impairing the study of the cytotrophoblast cell population. Therefore, we chose to study AEA effects on the cytotrophoblast cell model, BeWo cells, and concluded that this eCB affected cell viability, results that were in agreement to those already described [20].

The antagonist of CB2 receptor was also able to reverse AEA-induced increase in caspase activities, loss of $\Delta\psi m$ and ROS/RNS generation. However, CB1 receptor is also involved in these effects since its antagonist attenuated all of them, with exception of the augment in caspase-3/7 activity. We previously reported that CB2 antagonist was also able to prevent the increase in caspase-3/7 and 9 activities and viability loss induced by the other major endocannabinoid, 2-AG, supporting the importance of this receptor in eCB-induced apoptosis in BeWo cells [22]. Our data revealed that the intrinsic apoptotic pathway is activated in the AEA-induced apoptosis, as supported by the loss of $\Delta\psi m$ and the increase in caspase-9 activity. The involvement of the intrinsic pathway in the apoptotic effects of AEA has been described in several cell types. In fact, we have previously reported that AEA induces apoptosis in rat decidual cells, through a CB1-dependent mechanism involving caspase-9 activation, loss of $\Delta\psi m$, ceramide accumulation and phosphorylation of p38 [10]. Furthermore, in glioma cells, AEA also induced cytochrome *c* release via CB1, dependently on lipid rafts [27]. In pheochromocytoma PC12 cells, AEA induced cytochrome *c* release through the activation of p38 and JNK pathways [28].

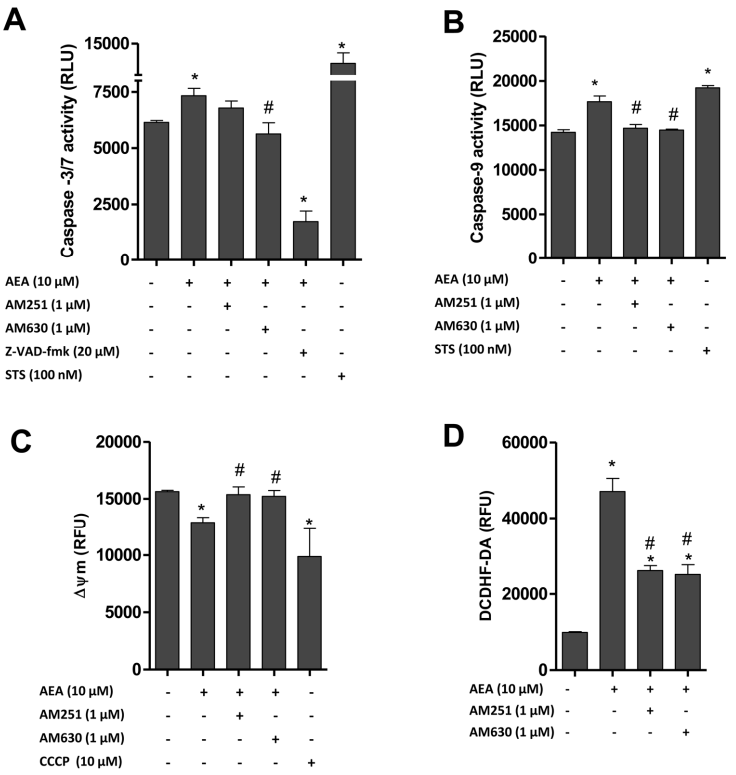

Figure 4. The influence of anandamide (AEA) in apoptotic markers and production of reactive species of oxygen and nitrogen (ROS/RNS) in BeWo cells. (A) After 36 h of treatment, AEA (10 μM) augmented the activity of caspases 3/7 and this effect was partially reversed by the CB2 antagonist AM630. (B) In comparison with untreated cells, AEA induced an increase in caspase 9 activity and both CB receptors antagonists partially reversed this effect. (C) AEA induced mitochondrial membrane potential ($\Delta\psi m$) loss, effect reversed by both CB1 and CB2 antagonists; CCCP, a depolarizing gent was used as a positive control. (D) AEA dramatically incremented the ROS/RNS production, after a 48h treatment and this effect was significantly reversed by both CB receptors antagonists. (*$p < 0.05$ vs. Control; #$p < 0.05$ vs. AEA 10 μM; Kruskal-Wallis test followed by Dunn's multiple comparison test). $DiOC_6$- 3, 3′-dihexyloxacarbocyanine iodide; CCCP-carbonyl cyanide m-chlorophenylhydrazone; DCDHF-DA- 2, 7-Dichlorodihydrofluorescein diacetate; STS-Staurosporine.

AEA increased caspase-8 activity via a CB receptor-dependent mechanism. It is important to mention that caspase-8 is involved in non-apoptotic cellular events such as proliferation, migration and differentiation [29]. This protease may be relevant for cytotrophoblast fusion and differentiation, though its role is not well established [30-32]. The activation of caspase-8 implies a dimerization and autocleavage at DISC. The homodimerization leads to full activation of procaspase 8 initiating the apoptotic process. However, the heterodimerization of procaspase-8 with the regulatory protein FLICE inhibitory protein (FLIP) limits procaspase-8 autoprocessing, allowing the initiation of a nonapoptotic pathways, mainly by the activation of the transcription factor NF-κB [29, 33]. Thus, caspase-8 may be an important molecule in the cell fate decisions between death and survival. Our results showed that NF-κB inhibitor protein, IκB-α, was upregulated in AEA-treated BeWo cells, indicating that the NF-κB pathway was not active and confirming the role of caspase-8 in the apoptotic process. In addition, we detected an increased formation of t-Bid. The cleavage of Bid by caspase-8 gives rise to t-Bid [34]. In this way, our data suggest that there is a crosstalk between the extrinsic and intrinsic apoptotic pathways, in AEA-induced apoptosis in BeWo cells. Other authors have already reported that AEA triggers the extrinsic apoptotic pathway. In fact, in cholangiocarcinoma cells, AEA caused apoptosis through a CB receptor-independent mechanism, by accumulation of ceramide and recruitment of Fas and FasL into lipid rafts [35]. More recently, these effects were attributed to the activation of GPR55 [36]. In Chang liver cells, AEA mediated cell death by activation of both extrinsic and intrinsic pathways, with upregulation of FasL, Bim and Bax, in a mechanism that also involved ceramide production, oxidative stress generation and phosphorylation of JNK and p38 [13].

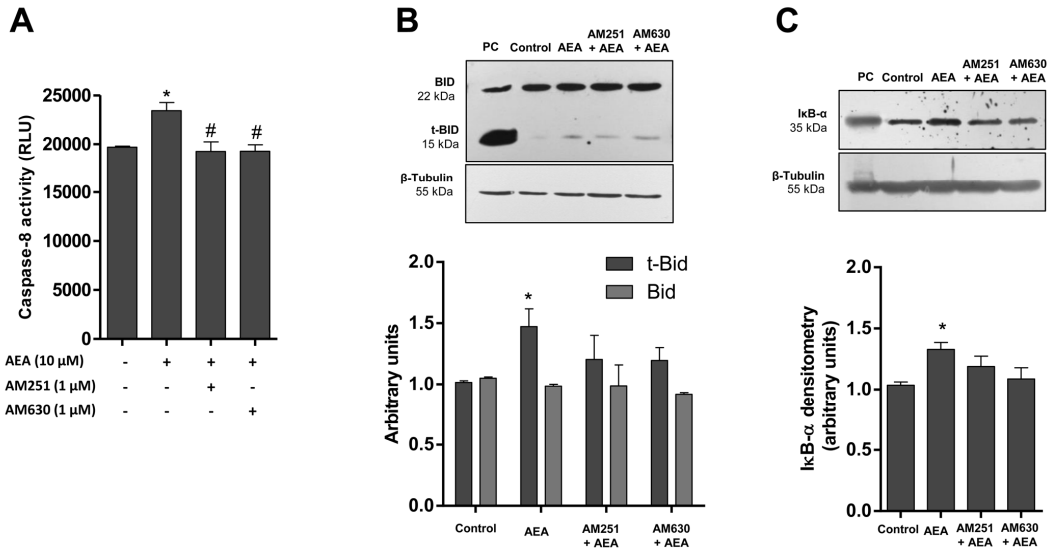

Figure 5. Impact of anandamide (AEA) in caspase-8 activity, cleavage of Bid and IκB-α expression in BeWo cells treated with 10 µM. (A) After 36 h, AEA increased caspase 8 activity, effect that was partially reversed by CB1 and CB2 antagonists. (B) After a 48 h treatment, AEA induced an increase of t-Bid formation and (C) increased the expression of the NF-κB regulatory protein, IκB-α, effects that were attenuated by both CB receptor antagonists. β-tubulin was used as loading control. BeWo cells treated with staurosporine (Bid/t-Bid) and HeLa cells extract (IκB-α) were used as positive controls (PC). (*$p < 0.05$ vs. Control; #$p < 0.05$ vs. AEA 10µM; Kruskal-Wallis test followed by Dunn's multiple comparison test).

It was previously demonstrated that both mitochondrial and death receptors pathways regulate the programmed cell death of trophoblasts [3, 37] and that caspases mediate apoptosis in villous cytotrophoblasts [38]. In addition, an abnormal expression of apoptotic proteins such as Bax, Bcl-2 or FasL in these cells is associated with pathological conditions like preeclampsia, HELLP syndrome, IUGR and hyperglycaemia [4-7, 39, 40]. Moreover, disturbances to pro-oxidant/antioxidant equilibrium during pregnancy may impair the biological function of proteins and lipids [41, 42] and are also associated with pregnancy complications like preeclampsia, intrauterine growth restriction (IUGR) and gestational diabetes [43-45].

In summary, this work showed that AEA induces apoptosis in human cytotrophoblasts and in a cytotrophoblast cell model, in the latter by triggering a CB receptor-dependent mechanism that involves both intrinsic and extrinsic apoptotic pathways. In this way, this work contributes to clarify the influence of AEA in cytotophoblast cells turnover and reinforces the relevance of a balanced endocannabinoid signalling for a successful placental development, since anomalies in apoptosis and oxidative stress have been associated with the aetiology of pregnancy-associated conditions like preeclampsia or IUGR.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

The authors thank Fundação para a Ciência e Tecnologia (FCT) for the PhD grant of Mariana Costa (SFRH/BD/70721/2010) and for the Post-doctoral grant of Bruno Fonseca (SFRH/BPD/72958/2010). The authors also thank to the Department of Obstetrics and Gynaecology of S. João Hospital Center.

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Manuscript III

2-Arachidonoylglycerol impairs human cytotrophoblast cells syncytialization: Influence of endocannabinoid signalling in placental development.

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Reprinted from Molecular and Cellular Endocrinology, 2014.
doi:10.1016/j.mce.2014.09.005.

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

2-Arachidonoylglycerol impairs human cytotrophoblast cells syncytialization: Influence of endocannabinoid signalling in placental development

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ARTICLE INFO

Article history:
Received 8 May 2014
Received in revised form 4 September 2014
Accepted 4 September 2014
Available online

Keywords:
2-arachinoylglycerol
Endocannabinoid signalling
Human cytotrophoblasts
Syncytialization
Placenta

ABSTRACT

A balanced cytotrophoblast cell turnover is crucial for placental development and anomalies in this process associated with gestational diseases. The endocannabinoid system (ECS) has emerged as a new player in several biological processes. However, its influence during placental development is still unknown. We report here the expression of the endocannabinoid 2-arachidonoylglycerol (2-AG) main metabolic enzymes in human cytotrophoblasts and syncytiotrophoblast. We also showed that 2-AG induced a decrease in placental alkaline phosphatase activity, human chorionic gonadotropin secretion and *Leptin* mRNA levels. Moreover, 2-AG reduced *glial cell missing 1* and *syncytin-2* transcription and the number of nuclei in syncytium. These effects were mediated by cannabinoid receptors and may result from 2-AG inhibition of the cAMP/PKA signalling pathway. Our data suggest that 2-AG may interfere with the biochemical and morphological differentiation of human cytotrophoblasts, through a CB receptor-dependent mechanism, shedding light on a role for the ECS in placental development.

1. Introduction

Development of the human placenta is a highly coordinated event that is crucial for a successful pregnancy outcome. The main specialized cell type of this organ is the trophoblast, which is composed of four cell populations: cytotrophoblasts (CTs), syncytiotrophoblast (ST), extravillous trophoblasts (EVTs) and giant trophoblast cells. CTs are proliferative, mononucleated cells that differentiate into STs and EVTs during the process of placentation. EVTs accomplish invasion which, ultimately, results in the anchoring of the placenta to the uterine wall and in the remodelling of maternal arteries, decreasing the resistance to blood flow. On the other hand, STs are multinucleated, non-proliferative cells that form a continuous layer lining the chorionic villi. They have a short lifespan and are in a constant renewal, dying by apoptosis and being replaced by new cells resulting from differentiation and fusion of CTs. The ST layer is in direct contact with maternal blood and is responsible for maternal–fetal gas and nutrient exchanges and fetal protection (Gude et al., 2004; Lunghi et al., 2007). Moreover, STs have the machinery necessary to produce hormones and proteins, such as human chorionic gonadotropin (hCG), oestrogens, human placental lactogen, placental growth hormone and leptin, that regulate feto-maternal physiology and metabolism and sustain pregnancy (Malassine and Cronier, 2002). Anomalies in placental development and CT differentiation have been related to gestational diseases like preeclampsia, intrauterine growth restriction and spontaneous miscarriage (Langbein et al., 2008; Lim et al., 1997; Ruebner et al., 2010).

Despite the crucial importance of CT differentiation for the process of placentation, the signalling pathways that orchestrate this process are not well clarified. Some authors argue that the activation of early stages of the apoptotic cascade, namely, the exposure of phosphatidylserine on the outer leaflet of plasma membrane and caspase 8 activation, are essential for CT differentiation (Gauster and Huppertz, 2010). The importance of cyclic AMP (cAMP) and protein kinase A (PKA) activation in this process is recognized (Guilbert et al., 2010; Rote et al., 2010).

The endocannabinoid system (ECS) has been suggested as an emergent intervener in physiologic and pathophysiologic cellular events in several organs and tissues. This system is constituted by the G-protein coupled cannabinoid receptors 1 and 2 (CB1, CB2), their endogenous ligands (the endocannabinoids [eCBs]) and the respective metabolic enzymes and transporters. Among the several agonists of CB receptors that have already been identified,

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http://dx.doi.org/10.1016/j.mce.2014.09.005
0303-7207/

anandamide (AEA) and 2-arachydonoylglycerol (2-AG) are the best studied and those whose biological relevance has been widely recognized. AEA and 2-AG are mainly synthesized from membrane phospholipids by *N*-arachidonoylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively, and are degraded by the cytosolic enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. eCBs are mainly produced "on demand" and have essentially an autocrine and paracrine action (see Fonseca et al., 2013b for review).

In the last years, eCBs have been identified as novel mediators in the complex signalling pathways that coordinate pregnancy events like implantation and decidualization (Taylor et al., 2010). However, there is a lack of evidence about the role of eCBs in placental development. In human placenta, CB receptors and AEA metabolic enzymes have already been described (Habayeb et al., 2008; Helliwell et al., 2004; Kenney et al., 1999; Park et al., 2003; Trabucco et al., 2009), but the role of 2-AG in this organ is still unknown, though the expression of its enzymatic machinery was reported in rat and baboon placentas (Brocato et al., 2013; Fonseca et al., 2012) and by our group in human cytotrophoblasts (Costa et al., 2014). In addition, we reported that 2-AG induces apoptosis in BeWo cells (a cytotrophoblast cell model), involving the apoptotic mitochondrial pathway, by a CB receptor-dependent mechanism (Costa et al., 2014).

In this work, we studied primary cultures of human cytotrophoblasts since these cells spontaneously differentiate and fuse into STs, in the presence of fetal bovine serum (Kliman et al., 1986). During differentiation, CTs aggregate and fuse, losing the expression of proteins that participate in the establishment of cell junctions, such as E-cadherin and desmoplakin. Furthermore, these differentiated cells express proteins such as placental alkaline phosphatase (pALP) and hCG. In this way, we investigated the presence of the main metabolic enzymes of 2-AG (DAGL-α and MAGL) in both human cytotrophoblasts and syncytiotrophoblast. In addition, we studied the impact of this endocannabinoid during the morphological and functional *in vitro* differentiation of human CTs into STs and the cellular mechanisms underlying 2-AG effects during these processes.

2. Materials and methods

2.1. Primary cultures of human cytotrophoblasts

All the procedures concerning human placental handling were performed in accordance with the Ethical Committee of Hospital S. João, Porto. In this study, we included normal term placentas (38–40 weeks of gestation) from Caucasian women living in the Porto region and aged 24–36 years old. For each assay, we isolated cytotrophoblasts from five different placentas. This isolation was performed using a modification of the Kliman's protocol, as previously described (Keating et al., 2007). Briefly, decidual tissue was removed and villous tissue collected from at least 10 different regions homogeneously distributed in the whole placenta. The major blood vessels were discarded by fine dissection. Then, the tissue was digested in a trypsin (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and DNAse (Sigma Aldrich Co. St Louis, MO, USA)-containing solution and the obtained cells were separated in a discontinuos percoll gradient. Cytotrophoblasts were collected and seeded in 24-well plates, 21 cm^2 dishes or 8-well chamber slides, at densities 1×10^6, 1×10^7 or 4.5×10^5, respectively, in DMEM/F12 medium (Sigma Aldrich Co. St Louis, MO, USA) supplemented with 10% (v/v) of fetal bovine serum (FBS) and an antibiotic–antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Sigma Aldrich Co. St Louis, MO, USA) and were incubated at 37 °C and 95% air/5% CO2 humidified atmosphere.

To characterize the enzymatic machinery involved in 2-AG metabolism, the cells were collected at 12 h and 72 h of culture, corresponding to two different stages of differentiation (cytotrophoblast and to syncytiotrophoblast cells, respectively), in TRIzol® reagent (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and RNA and protein were extracted according to the manufacturer's instructions.

For the studies of cell viability and LDH release, cells were treated with 2-AG (dissolved in ethanol) for 48 h, in two different times of culture: at 12 h, to study the effects of this eCB during CT differentiation, and at 72 h, to investigate its effect in the syncytiotrophoblast. For the experiments of the differentiation markers assessment, cells were treated with 2-AG at 12 h of culture, for 48 h, to assess its effect during the differentiation process.

2.2. RT-PCR analysis

The assessment of *MAGL* and *DAGL-α* gene transcription was carried out by RT-PCR. RNA was quantified in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), its quality evaluated with the Experion RNA StdSens Kit (Bio-Rad Laboratories, USA) and analysed with Experion analytical software (Bio-Rad Laboratories, USA). cDNA was obtained by reverse transcription of RNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, USA) and it was subsequently amplified with specific primers, using KAPA SYBR® FAST qPCR Master Mix 2x Kit (Kapa Biosystems, Woburn, MA, USA) in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, USA), according to the kit protocol. Table 1 resumes primer sequences and RT-PCR conditions. The specificity of PCR product amplification was assessed by analysis of the melting curve. Gene expression of *DAGL-α* and *MAGL* was normalized with two housekeeping genes (β-actin and succinate dehydrogenase subunit A, SDHA) and their analysis was achieved by the calculation of ΔΔCT values.

2.3. Western Blot analysis

Cells were harvested in lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% Triton™ X-100), at 4 °C, and the protein fraction was obtained by centrifugation at 14,000 *g* for 10 min, at 4 °C. For PKA phosphorylation analysis, cells were treated for 15 minutes with 2-AG, in the absence or presence of CB receptor antagonists. Protein extracts were loaded in 10% SDS-PAGE and transferred onto nitrocellulose membranes. Then, nonspecific binding sites were blocked and membranes were incubated with anti-MAGL, anti-DAGL-α or phospho-PKA α/β/γ cat Thr^{198} (Santa Cruz Biotechnology, USA; 1:100; rabbit) primary antibodies overnight, at 4 °C. Membranes were incubated at room temperature, for 1 h, with peroxidase-conjugated secondary antibody 1:1000 (anti-rabbit; Vector Laboratories, CA, USA), washed and exposed to a chemiluminescence detection kit (Super Signal West Pico; Pierce, Rockford, USA) and then to an X-ray film (Kodak XAR; Eastman Kodak, Rochester, NY). Rat brain was used as a positive control. For loading control, membranes were stripped and reincubated with anti-β-tubulin antibody (Santa Cruz Biotechnology, USA; 1:1000; rabbit). The signal intensity of DAGL-α, MAGL and p-PKA was quantified by densitometry (BIO-PROFIL Bio-1D2; Vilber Lourmat, Marne-la-Vallée, France) and the results expressed in arbitrary units, after normalization for the corresponding β-tubulin band.

2.4. Immunocytochemistry and Immunohistochemistry

For immunohistochemistry, deparaffined slides of human placenta (4 μm thick) were used. For immunocytochemistry analysis, cells were seeded in 8-well chamberslide and fixed with cold methanol at 12 h or 72 h of culture. The expression of proteins was

Table 1
Primer sequences and RT-PCR conditions used to assess the gene expression of diacylglycerol-α (DAGL-α), monoacylglycerol lipase (MAGL), leptin, glial cell missing 1 (GCM-1), syncytin-1 and syncytin-2, succinate dehydrogenase subunit A (SDHA).

Gene	GenBank	Primer sequence (5′-3′)	Annealing temperature	Amplicon length	Melting temperature	Ref.
hDAGL-α	NM_006133.2	Sense:TGCTCTTCGGCCTGGTCTAT Anti-sense:CGCATGCTCAGCCAGATGAT	61 °C	130 bp	85.6 °C	(Ludanyi, et al. 2008)
hMAGL	BC000551.2	Sense:CAAGGCCCTCATCTTTGTGT Anti-sense:ACGTGGAAGTCAGACACTAC	57 °C	162 bp	85.5 °C	(Ludanyi, et al. 2008)
hGCM-1	NM_003643.3	Sense: GACCAGGTCTCTTCCAGGTG Anti-sense:ACTACCAGGCAATTGGACGC	59 °C	88 bp	77.3 °C	
Syncytin-1 (HERVW-1)	NM_014590.3	Sense:GAAGTGTGCCAGAGAAATAATCC Anti-sense:CCAGTCTTCTCCAGGGTAATG	59 °C	87 bp	77.9 °C	
Syncytin-2 (HERVFRD-1)	NM_207582.2	Sense:CTTCCCTAGTGCCATTACAGT Anti-sense:TTGGGATCACCTTACTTTGAGG	60 °C	215 bp	80.6 °C	
hLeptin	NM_000230.2	Sense:TGCGGATTCTTGTGGCTTTG Anti-sense:CTGACTGCGTGTGTGAAATGT	60 °C	133 bp	77.6 °C	
hβ-actin	NM_001101.3	Sense:AACTCCATCATGAAGTGTGACG Anti-sense:GATCCACATCTGCTGGAAGG	60 °C	234 bp	85.5 °C	
hSDHA	NM_004168.2	Sense:AAACCAAACGCTGGGGAAGA Anti-sense:CTGAGTCGCAGTTCCGATGT	58 °C	89 bp	74.8 °C	

analysed using an avidin–biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ABC kit, Vector Laboratories, CA, USA). The non-specific binding sites were blocked and slides were incubated with anti-DAGL (1:100) or anti-MAGL (1:100) antibodies at 4 °C, overnight, followed by incubation with biotinylated secondary antibody and further incubation with Vectastain ABC-AP reagent, according to the manufacturer's instructions. The reaction was developed with Sigma Fast RedTM tablets (Sigma Aldrich Co, St. Louis, USA). The counterstain was performed using Mayer's haematoxylin solution (Sigma Aldrich, St. Louis, USA) and slides were mounted in Aquamount medium (BDH Laboratory Supplies, Poole, England). Negative controls were performed by the replacement of the primary antibodies by rabbit IgG.

2.5. Cell viability and LDH release

Cytotrophoblast cells were plated in 24-well plates and treated with 2-AG (1–20 μM) at 12 h and at 72 h of culture, for 48 h. The effects of this eCB on cell viability were monitored by MTT assay. In addition, we assessed the effects of the CB receptor antagonists, AM251 and AM630 (1 μM; vehicle ethanol) (Tocris Bioscience; Bristol, UK), in the absence or presence of 2-AG (10 μM), for 48 h, starting the incubation at 12 h of culture. The yellow tetrazole MTT (0.5 mg/ml final concentration) (Sigma Chemical Co, St. Louis, MO, USA) was added and cells were incubated at 37 °C for 3 h. The resultant purple formazan was dissolved in a solution of dimethylsulfoxide (DMSO):isopropanol (3:1) and spectrophotometrically quantified at 540 nm, using a Multiscan Ascent microplate reader. The release of the enzyme lactate dehydrogenase (LDH) to the culture medium, as a cytotoxicity marker, was assessed by the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

2.6. Ecto-placental alkaline phosphatase (pALP) activity assay

Cytotrophoblast cells were plated in 24-well plates and then treated at 12 h of culture with 2AG (1–20 μM), for 48 h. For the study of the involvement of CB receptors in 2-AG effects, cells were pre-exposed, for 30 minutes, to 1 μM of CB1 and CB2 antagonists, AM251 and AM630, respectively. At the end of the incubation period, the assay of ecto-pALP activity was performed as described by Keating (Keating et al., 2009). Briefly, cells were rinsed with buffer and then incubated at 37 °C for 1 h with the pALP substrate *p*-nitrophenilphosphate, 2.5 mM (Sigma Chemical Co, St. Louis, MO, USA), in the presence of 100 mM $MgCl_2$ (Sigma Chemical Co, St. Louis, MO, USA). The reaction was stopped by the addition of ice-cold 0.02 M NaOH solution to the extracellular medium, and the absorbance quantified at 405 nm, in a Multiscan Ascent microplate reader. The *p*-nitrophenol (pNP) formed was determined by interpolation in a calibration curve of pNP (Sigma Chemical Co, St. Louis, MO, USA). Cells were lysed and cell protein was quantified by the Bradford assay. The incubation with L-Phenylalanine (2 mM; Sigma Chemical Co, St. Louis, MO, USA), a pALP inhibitor, for 30 minutes before the addition of pALP substrate was used as a control. Equimolar concentrations of the vehicle (ethanol 0.1%) have no effects on cytotrophoblast cells (data not shown). The results were expressed in nmol of *p*-nitrophenol/mg of protein/minute. The final results were expressed in relative values, in comparison with the untreated cells (control). For the control of the spontaneous differentiation of cytotrophoblasts into syncytiotrophoblast, ecto-pALP was assessed at different times of culture (12, 72 and 120 h).

2.7. Quantification of secreted β-hCG

The cells were treated in the same conditions as referred for the ecto-pALP assay. After a 48 h-treatment with 2-AG (10 μM), cell culture medium was collected, centrifuged and stored at –80 °C. The impact of 2-AG in hCG production was evaluated by the quantification of the β subunit of this hormone in cell supernatants by ELFA (enzyme linked fluorescent assay) with the VIDAS HCG kit (bioMérieux SA, Marcy l'Etoile, France) using the mini-VIDAS autoanalyser (bioMérieux SA, Marcy l'Etoile, France), according to manufacturer's protocol. The results were standardized with total protein content, calculated as mIU/mg of protein and expressed as relative values compared to the control.

2.8. Evaluation of leptin, glial cell missing 1 (GCM-1), HERVW-1 (syncytin-1) and HERVFRD-1 (syncytin-2) gene expression by RT-PCR

The cells were seeded in 6 well plates and treated as described for the other assays. At the end of experiment, cells were collected in TRIzol® Reagent. The assessment of RNA quality, cDNA synthesis, gene amplification and semi-quantification by qPCR were performed as described above. Primer sequences and qPCR conditions are summarized in Table 1.

2.9. Analysis of cytotrophoblast cell fusion

For the assessment of the effect of 2-AG on cytotrophoblast cell fusion, the formation of the syncytiotrophoblast was evaluated by

the analysis of E-cadherin expression. Cells were seeded in chamberslides and were processed as described above. After treatment with 10 μM of 2-AG for 48 h, cells were washed with PBS and fixed with 4% paraformaldehyde. Unspecific binding sites were blocked and cells were incubated overnight, at 4 °C, with the primary antibody anti-E-cadherin (1:200; BD Biosciences, San Jose, Ca, USA). Cells were washed with PBS and incubated with FITC conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, USA; 1:200) for 1 h, at room temperature. Finally, slides were mounted in Fluoroshield™ containing DAPI (Sigma Chemical Co, St. Louis, MO, USA) and analysed in a confocal microscope, with laser excitation at 405 nm and 488 nm (Leica SP2 AOBS SE, Leica Microsystems, Wetzlar, Germany). The number of nuclei in syncytium was compared with the total number of nuclei and the resultant ratio expressed in percentage. For this analysis, a syncytium was defined as three or more nuclei inside a cytoplasm and 10 random fields were considered and counted for each treatment condition.

2.10. Cyclic AMP quantification assay

Cells were seeded in 96-well white plates and treated with 2-AG for 15 minutes, in the absence or presence of CB receptor antagonists and of pertussis toxin (PTX) 40 ng/ml (Sigma Aldrich Co. St Louis, MO, USA), a Gi protein inhibitor. cAMP levels were then assessed with the cAMP-glo™ Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions.

2.11. Statistical analysis

Statistical analysis was carried out by t-test or one or two-way ANOVA, followed by the Bonferroni post-hoc test to make pairwise comparisons of individual means when significance was indicated (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). At least five placentas were studied and the experiments were performed in triplicate. Data were expressed as mean SEM and differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Expression of the main enzymes involved in 2-AG biosynthesis and degradation in human cytotrophoblasts and syncytiotrophoblast

Gene transcription of the major enzymes that synthesize and hydrolyze 2-AG, DAGL-α and MAGL respectively, was investigated by RT-PCR (Fig. 1A). The transcript levels of *DAGL-α* and *MAGL* were similar in undifferentiated cytotrophoblasts (12 h of culture) and in differentiated cells (72 h of culture). Western blot and immunocytochemistry analysis corroborated RT-PCR results, revealing that

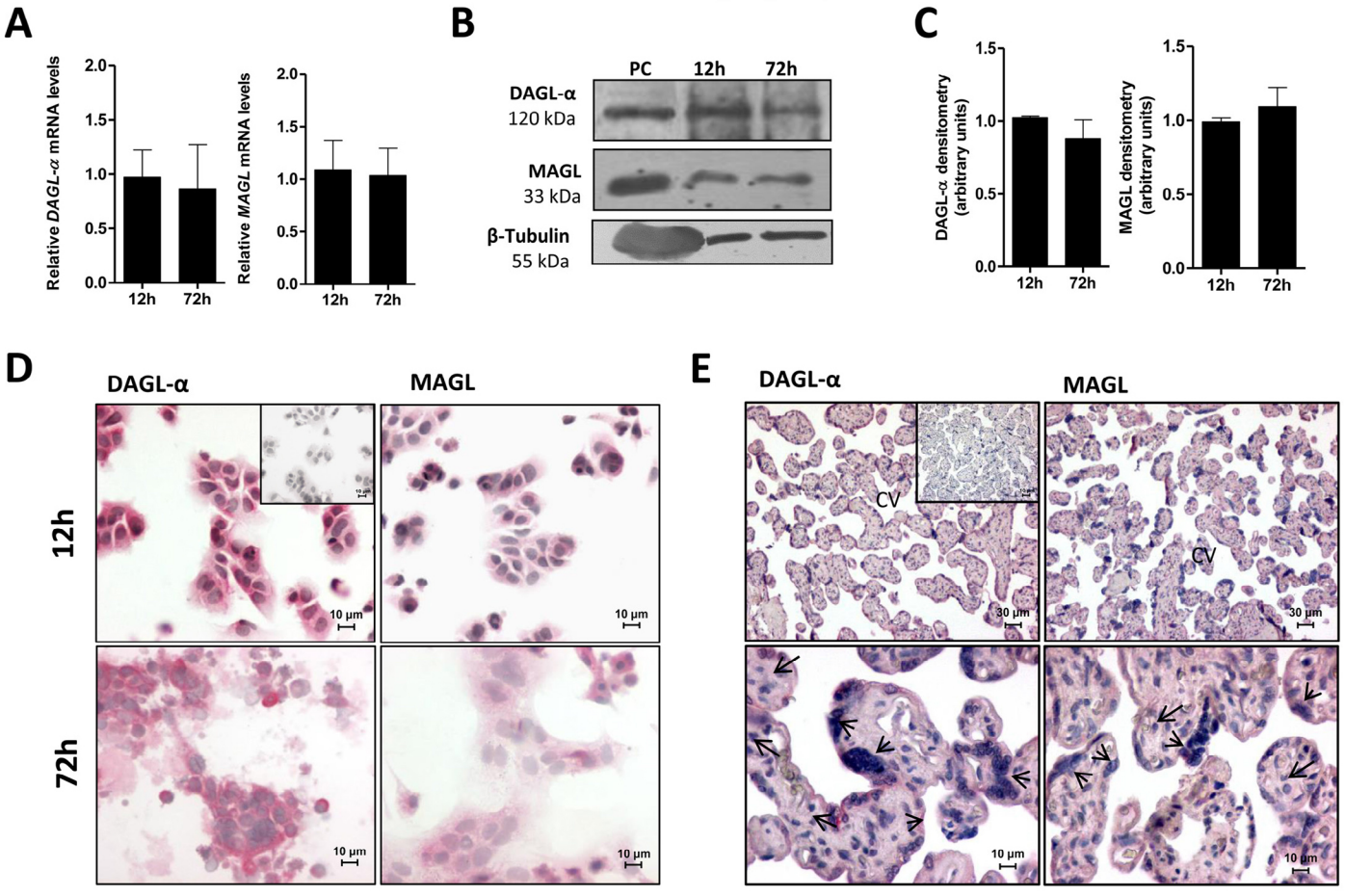

Fig. 1. Expression of the main metabolic enzymes of 2-arachidonoylglycerol (2-AG), diacylglycerol-α (DAGL-α) and monoacylglycerol lipase (MAGL), in human cytotrophoblasts at 12 and 72 h of culture. (A) mRNA levels of *DAGL-α* and *MAGL* were similar in both stages of differentiation, which is also observed in the translated proteins, as revealed by Western blot analysis by densitometry (B, C); β-tubulin was used as loading control. Rat brain homogenates were used as positive control (PC). (D) Immunostaining revealed a positive signal for DAGL-α and MAGL in the cells at 12 and 72 h of culture. (E) Immunohistochemistry showed that both enzymes are expressed in chorionic villi, in the CTs and ST; arrows and arrowheads denote the CTs and ST, respectively. The squares inserted in D and E represent a negative control. CV, chorionic villi; CT, cytotrophoblasts; ST, syncytiotrophoblast.

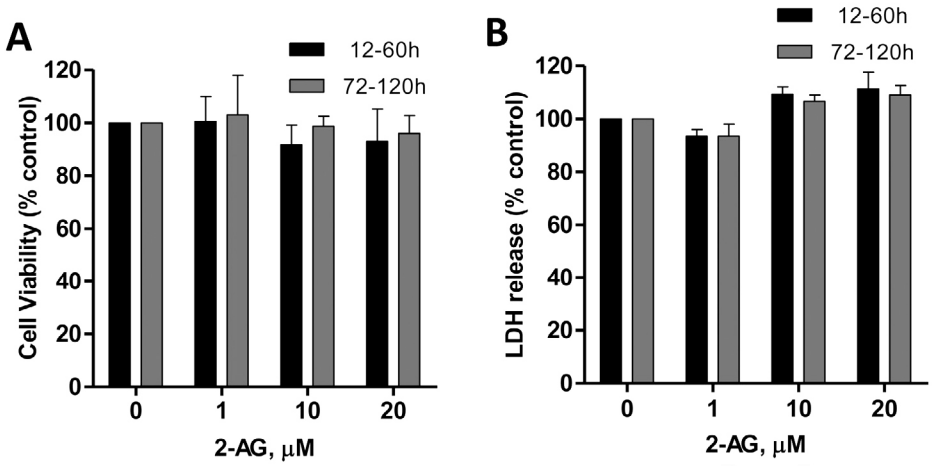

Fig. 2. Effects of 2-arachidonoylglycerol (2-AG) in trophoblast cells' viability (MTT assay). Cells were treated with different concentrations of 2-AG for 48 h at two different times of culture: at 12 and 72 h, to assess the effects on viability during the differentiation process and in the post-differentiation period, respectively. None of the tested concentrations was able to decrease cell viability (A) or induce LDH release to the culture medium (B).

both enzymes are expressed in the cytoplasm of cytotrophoblasts and syncytiotrophoblast (Figs 1B–D). Moreover, immunohistochemistry of placental sections revealed that both enzymes are expressed in chorionic villi (Fig. 1E).

3.2. *Effects of 2-AG in cytotrophoblast cell viability and LDH release during and after differentiation into syncytiotrophoblast*

To investigate the effects of 2-AG in CT differentiation, cells in the two different times of culture (12 and 72 h) were incubated with this eCB (1–20 μM), for 48 h. Treatment with 2-AG did not alter cell viability, as assessed by the MTT assay neither extracellular LDH levels, suggesting that this eCB is not cytotoxic, in the studied conditions (Fig. 2A, B).

3.3. *Impact of 2-AG in ecto-placental alkaline phosphatase (pALP) activity during the differentiation period*

The placental isoform of alkaline phosphatase is essentially expressed by the syncytiotrophoblast and its activity increases proportionally with the time of culture (Keating et al., 2009). Thus, this enzyme is frequently used as a biochemical marker to evaluate the *in vitro* differentiation of human cytotrophoblasts. The activity of this ecto-enzyme was assessed in cells treated at 12 h of culture with 2-AG (1–20 μM), for 48 h (Fig. 3A). We could observe that 10 and 20 μM were able to decrease significantly the ecto-pALP activity. The lowest 2-AG concentration that was able to decrease the ecto-pALP activity, 10 μM, was chosen for the experiments. To elucidate the mechanism behind this effect, cytotrophoblast cells were incubated with CB receptor antagonists. It was observed that the

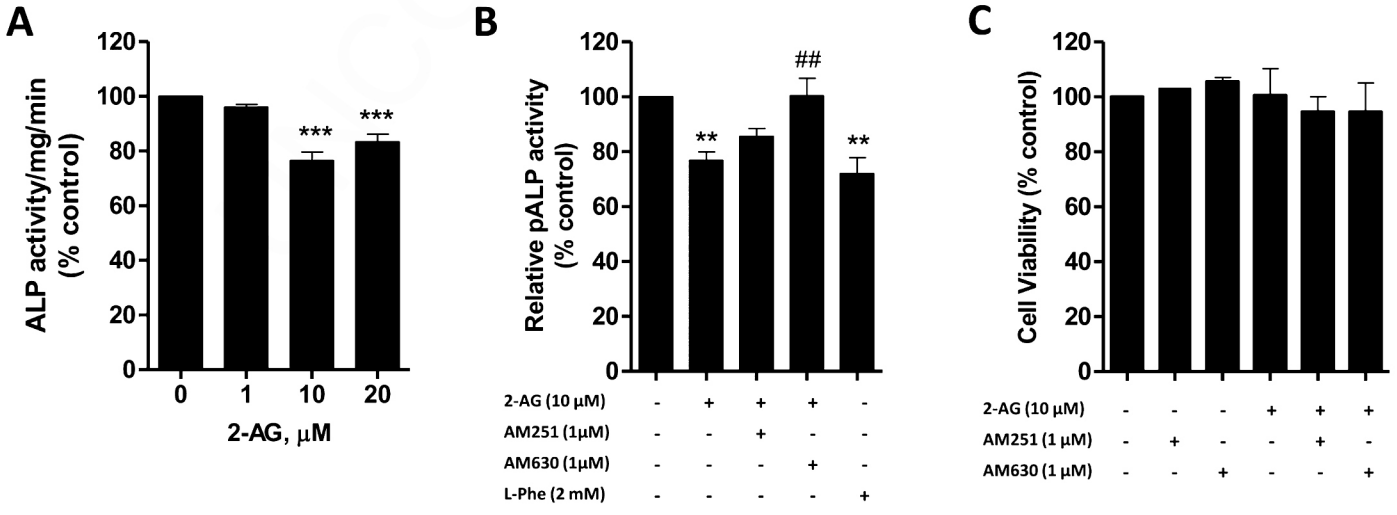

Fig. 3. Effects of a 48 h-treatment with 2-arachidonoylglycerol (2-AG) in placental alkaline phosphatase (pALP) activity in human cytotrophoblasts treated at 12 h of culture. (A) 2-AG decreased pALP activity at 10 and 20 μM. (B) The reduction in pALP activity induced by 10 μM of 2-AG was reversed by AM630, a CB2 antagonist but not by the CB1 antagonist, AM251. (C) CB receptor antagonists, AM251 and AM630 (1 μM), did not decrease cytotrophoblast cell viability, in the absence or presence of 2-AG. L-Phenylalanine (L-Phe) was used as control for ecto-pALP activity (*** $p < 0.001$; ** $p < 0.01$ vs. Control; ## $p < 0.01$ vs. 2-AG 10 μM).

CB2 antagonist, AM630, reversed the 2-AG-induced reduction in ectopALP activity. The CB1 antagonist (AM251) only attenuated 2-AG effects by 9% (Fig. 3B). To verify that these effects were not due to fluctuations in cell viability, we examined the effects of CB receptor antagonists in the absence or presence of 2-AG (10 μM). Again, these conditions were harmless for cytotrophoblast viability (Fig. 3C).

3.4. Effects of 2-AG in hCG secretion

The placental hormone hCG is essentially produced by the syncytiotrophoblast and is crucial for different cellular processes required for a proper pregnancy outcome. The quantification of β subunit of this hormone revealed that 2-AG (10 μM) induced a notorious decrease (43%) in its secretion, after 48 h-treatment (Fig. 4A). Preincubation with both CB receptor antagonists reversed 2-AG effects in 28% for AM251 and 27% for AM630, suggesting that CB1 and CB2 are implicated in the mechanism involved in 2-AG-induced decrease in hCG secretion.

3.5. Leptin gene transcription in 2-AG-treated cytrophoblasts

In placenta, leptin is essentially produced by the syncytiotrophoblast. The evaluation of mRNA levels revealed that cytotrophoblasts treated with 2-AG (10 μM) for 48 h presented a 33% decrease in *Leptin* gene transcription, in comparison with untreated cells (Fig. 4B). This decrease was significantly reversed by both AM251 and AM630, suggesting that the 2-AG effect in *Leptin* gene transcription is CB receptor-dependent.

3.5. Role of 2-AG in the morphological differentiation of human cytotrophoblasts

E-cadherin is a transmembrane protein required for cell–cell adhesion. Its expression decreases in parallel with the differentiation of the mononuclear cytotrophoblast cells into the multinucleated syncytiotrophoblast. Human trophoblasts express two fusogenic retroviral envelope proteins, syncytin-1 and syncytin-2, which are encoded by HERVW-1 and HERVFRD-1, respectively. These fusogenic proteins participate in the formation of the syncytium and its transcription is regulated by GCM-1. To assess 2-AG interference in morphological differentiation of cytotrophoblast cells, we studied the transcription of *GCM-1* and also of HERVW-1 and HERVFRD-1 in cells treated with 2-AG (10 μM) and CB receptor antagonists. 2-AG decreased the mRNA levels encoding for GCM-1 (Fig. 5A) and for syncytin-2 (Fig. 5C), though no significant effects were detected for syncytin-1 (Fig. 5B). The effects in GCM-1 and syncytin-2 transcription were reversed by both CB receptor antagonists. We also observed that 2-AG treatment reduced the number of syncytium in 22%, as shown by the decrease in the number of nuclei within the same cell boundary, represented by the green staining for E-cadherin (Figs 5D, E). CB1 or CB2 receptor antagonists were also able to reverse the decrease in syncytialization (Fig. 5D).

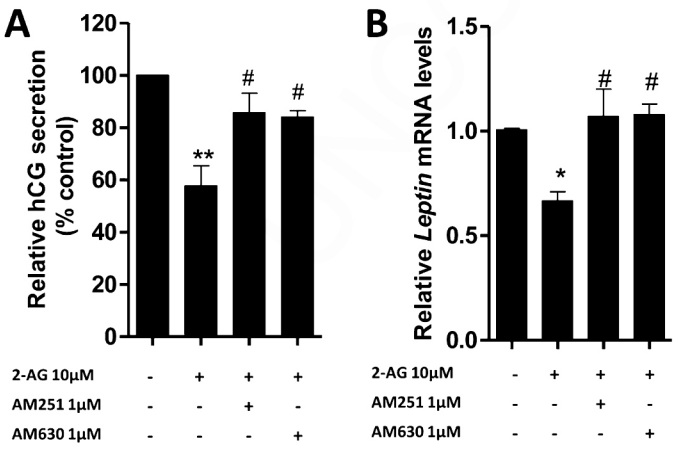

Fig. 4. Effects of 2-arachidonoylglycerol (2-AG) in the human chorionic gonatropin (hCG) secretion and *Leptin* mRNA transcription by human cytotrophoblasts. (A) Treatment with 2-AG (10 μM) for 48 h induced a notable decrease in hCG secretion and both CB receptor antagonists were able to reverse this reduction. (B) RT-PCR analysis revealed that *Leptin* transcript levels were decreased in 2-AG-treated cells, this effect being significantly reversed by CB1 and CB2 antagonists. (*$p < 0.05$, **$p < 0.01$ vs. Control; #$p < 0.05$ vs. 2-AG 10 μM).

3.6. Influence of 2-AG in the cAMP/PKA pathway during human cytotrophoblasts differentiation

The cyclic AMP/protein kinase A (cAMP/PKA) pathway is important for the syncytialization process. We concluded that 2-AG (10 μM) decreased the levels of cAMP, an effect that was reversed by both CB receptor antagonists and also by PTX (Fig. 6A). In addition, 2-AG diminished the phosphorylation of PKA, an effect that was also reversed by CB receptor antagonists (Figs 6B, C).

4. Discussion

The development of placenta is a critical event for the pregnancy success. In fact, impairments in the process of placentation resulting from anomalies in cytotrophoblast proliferation or differentiation have been associated with pregnancy complications (Langbein et al., 2008; Lim et al., 1997; Ruebner et al., 2010). The role of endocannabinoids during gestation is not well clarified but an unbalanced endocannabinoid signalling has been related to gestational complications, such as miscarriages (Fonseca et al., 2013a; Taylor et al., 2010). The endocannabinoid 2-AG modulates implantation (Wang et al., 2007) and decidualization (Fonseca et al., 2010) in rodents; its importance in human placental development has been poorly investigated, though our group described that 2-AG induces apoptosis in BeWo cells (Costa et al., 2014).

We have previously demonstrated the expression of the main enzymes that synthesize and hydrolyze 2-AG (DAGL-α and MAGL, respectively), in human cytotrophoblasts (Costa et al., 2014). In this work, we report that both human cytotrophoblasts and syncytiotrophoblast express these enzymes in similar levels, indicating that the expression of these molecules does not fluctuate with the differentiation status of trophoblasts. These enzymes are required to locally regulate 2-AG levels and their presence suggests that 2-AG function may be relevant for placentation. Thus, we investigated a possible role for 2-AG during *in vitro* differentiation of cytotrophoblast into the syncytiotrophoblast, by the assessment of biochemical and morphological differentiation markers.

We observed that exposure of cytotrophoblasts to 2-AG induced a decrease in pALP activity, a remarkable reduction in hCG secretion and a decrease in leptin transcripts, three relevant markers of cytotrophoblast differentiation. This was accompanied by an impairment in cytotrophoblast fusion, as revealed by the diminished *GCM-1* and *syncytin-2* mRNA levels and E-cadherin staining. Importantly, the observed decrease in the levels of differentiation markers is not due to a reduction in cell viability. We also demonstrated that the decrease in hCG secretion, *Leptin*, *GCM-1* and *syncytin-2* mRNA levels and in the number of nuclei in syncytium was partially reversed by both CB1 and CB2 antagonists. However, the effect of 2-AG in pALP activity was only significantly reversed by the CB2 antagonist.

CB receptors are G-protein coupled receptors and are mainly coupled to $G_{i/0}$ subunits, whose activation triggers several signalling pathways, including the inhibition of adenylyl cyclase. Consequently, the decrease in cAMP levels leads to an inhibition of

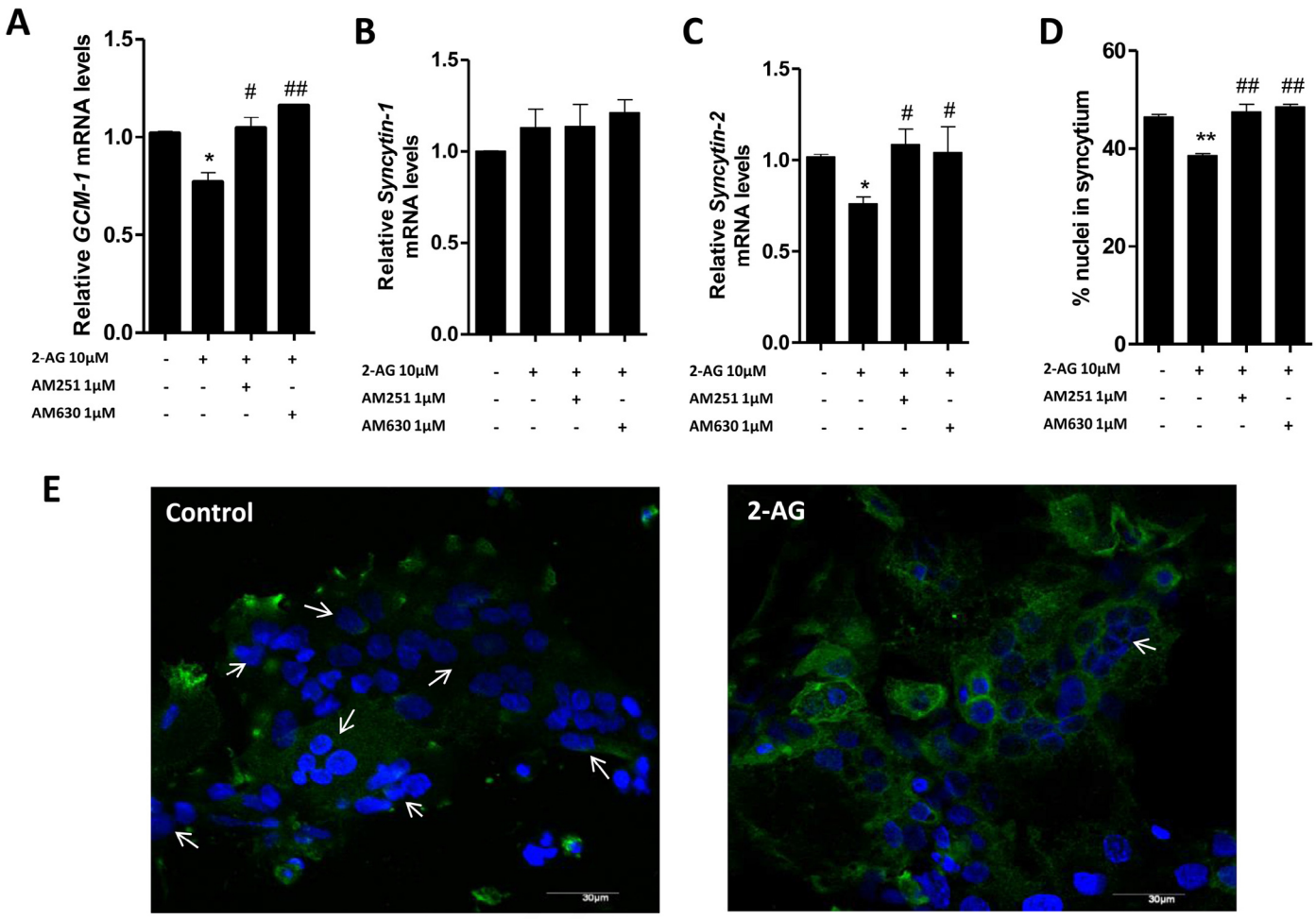

Fig. 5. Impact of 2-arachidonoylglycerol (2-AG) in morphological differentiation of cytotrophoblast cells treated for 48 h. RT-PCR analysis showed that 2-AG decreased the transcription of glial cell missing 1 (GCM-1) (A) and syncytin-2 (C), these effects being mediated by both CB receptors. No significant effects were detected in syncytin-1 transcription (B). (D) Immunofluorescence for E-cadherin revealed that 2-AG decreased the ratio number of nuclei in syncytium vs. the total number of nuclei; pre-treatment with CB1 and CB2 antagonists reversed this effect. (E) Immunofluorescence images for E-cadherin (green) in cells counterstaining with DAPI (blue), which are representative of 2-AG effects in the formation of multinucleated syncytiotrophoblast; arrows denote a syncytium. (*$p < 0.05$, **$p < 0.01$ vs. Control; #$p < 0.05$, ##$p < 0.01$ vs. 2-AG 10 µM).

cAMP-dependent protein kinase A (PKA) pathway (Demuth and Molleman, 2006). The importance of the cAMP/PKA cascade in CTs' morphological and biochemical differentiation is known for years. An increase in cAMP levels is required for the differentiation process and for the synthesis of hCG, in a mechanism that leads to PKA activation and phosphorylation of cAMP response element binding protein (CREB) (Keryer et al., 1998a, 1998b; Knofler et al., 1999; Milsted et al., 1987). In BeWo cells, a crosstalk between cAMP/PKA and mitogen-activated protein kinase (MAPKs) pathways was described to promote CT differentiation, by increasing hCG biosynthesis and expression of fusogenic genes (Delidaki et al., 2011). However, besides the cAMP-activated pathways, it was suggested that other cellular mechanisms may regulate hCG secretion in BeWo cells (Orendi et al., 2010). Our data revealed that 2-AG inhibits cAMP/PKA signalling pathway. Hereupon, it is likely that 2-AG-induced inhibition of CT differentiation results from the activation of CB receptors, which will consequently decrease cAMP levels, diminishing PKA activity. Moreover, we showed that 2-AG effects on cAMP levels were reversed by PTX, an inhibitor of Gi protein, which supports that CB receptors are coupled to this G protein subunit in cytotrophoblast cells and explains the decreased levels of cAMP after 2-AG treatment.

The multiple functions of hCG during pregnancy are well documented. In fact, it participates in several pregnancy events, such as stimulation of progesterone synthesis by corpus luteum, angiogenesis, immunoprotection and CT fusion and differentiation (Cole, 2012). In this way, disruption in the endocannabinoid signalling may interfere with these processes, due to the reduction of hCG production. Additionally, hCG enhances the production of leptin by the human ST, through a mechanism that involves an interplay between cAMP and MAPKs (Ge et al., 2011). This hormone has pleotropic effects in pregnancy, such as angiogenesis, stimulation of hCG secretion, CT proliferation, blastocyst-endometrium communication and implantation (Henson and Castracane, 2006). Our results revealed that 2-AG decreased leptin transcripts, suggesting that this eCB also affects the production of this protein. A linkage between leptin and ECS has already been described. In fact, this cytokine activates the promotor of FAAH in human T lymphocytes, reducing AEA levels (Maccarrone et al., 2003). Also, in leptin knockout mice, levels of AEA and 2-AG are elevated in uterus, in comparison with wild-type mice (Maccarrone et al., 2005).

Placental alkaline phosphatase is a widely used marker of syncytialization, since its expression is mainly restricted to STs. The physiological role of pALP is not clear, but there are evidences of

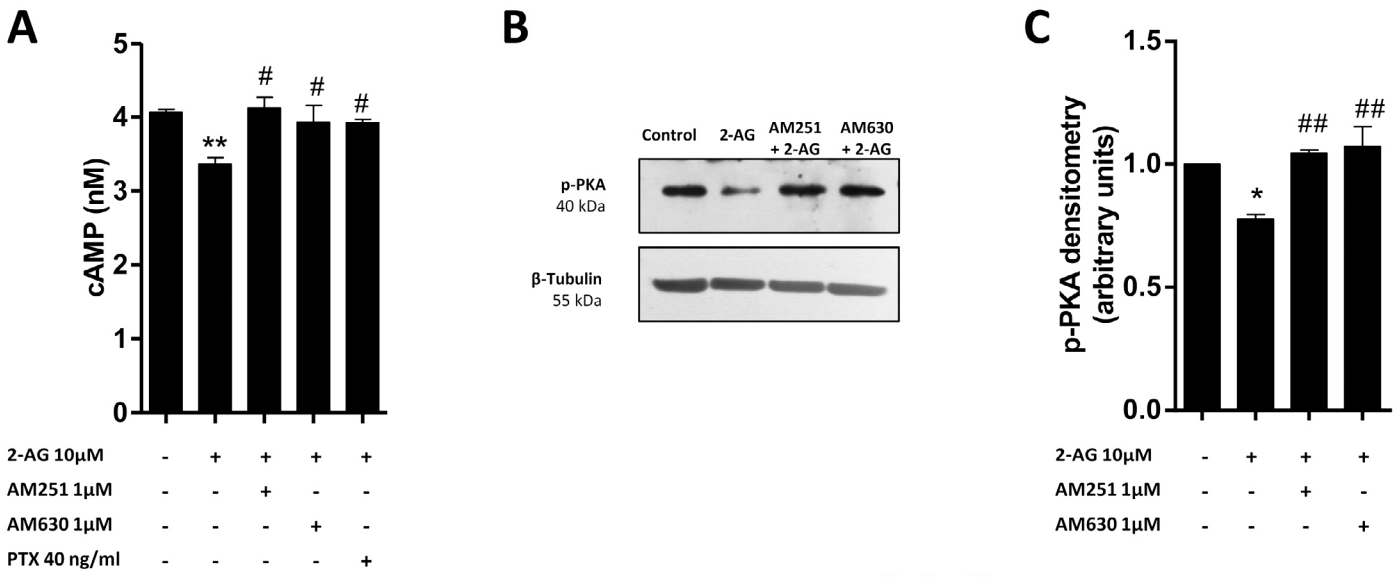

Fig. 6. 2-Arachidonoylglycerol (2-AG) effects in cyclic AMP/ protein kinase A (cAMP/PKA) pathway. (A) 2-AG decreased cAMP levels, an effect that was reversed by both CB receptor antagonists and also by pertussis toxin (PTX), inhibitor of Gi protein. (B) Representative Western blot of 2-AG effects showing a decrease in the phosphorylation of PKA through a CB receptor dependent mechanism, as revealed by the densitometry analysis (C). (*$p < 0.05$, **$p < 0.01$ vs. Control; #$p < 0.05$, ##$p < 0.01$ vs. 2-AG 10 μM).

its participation in the transport of maternal IgG to the fetus (Makiya and Stigbrand, 1992a, 1992b). Its activity is decreased in cytotrophoblasts isolated from intrauterine growth restriction placentas (Keating et al., 2009). Our data showed that 2-AG reduced pALP activity through CB2 activation, suggesting an impairment in CTs' biochemical differentiation. Since the expression of this enzyme seems also to be regulated by cAMP-activated mechanisms (Orendi et al., 2010), the 2-AG-induced decrease in pALP activity may also be a result of the reduction in cAMP levels.

Since 2-AG interferes with the levels of some important proteins for the gestational course, it is suggested that deregulations in endocannabinoid signalling may negatively impact on placental development and consequently may participate in the pathophysiological mechanisms of pregnancy complications.

Moreover, our results showed that 2-AG also affects the cytotrophoblast cells fusion by a CB receptor-dependent mechanism, as observed by the reduction in the mRNA levels of the transcription factor GCM-1 and the fusogenic protein syncytin-2 and also in the number of syncytialized cells, though with no effects on syncytin-1 transcription. Syncytin-1 and -2 participate in the cell fusion and formation of the syncytiotrophoblast. Since it regulates the transcription of the syncytins, GCM-1 is crucial for the trophoblast cell fusion process (Baczyk et al., 2009; Liang et al., 2010; Yu et al., 2002). Syncytin-2 is important for human trophoblast cells fusion (Benaitreau et al., 2010; Chen et al., 2008; Vargas et al., 2009) and anomalies in its expression reflects the impaired trophoblast differentiation observed in some pathological conditions (Malassine et al., 2008; Vargas et al., 2011). It is described that cAMP regulates the activation of GCM-1 either by PKA-dependent (Knerr et al., 2005; Lin et al., 2011) or independent (Chang et al., 2011) mechanisms in BeWo cells. 2-AG-induced impairments in GCM-1 and syncytin-2 transcription, through the activation of both CB1 and CB2 receptors, will imply a negative impact in morphological syncytialization as corroborated by the E-cadherin staining results.

Nevertheless, our results were obtained with *in vitro* studies performed with supraphysiological concentrations of 2-AG, which are in agreement with those used in *in vitro* studies performed by others working in the endocannabinoids research field. Further studies are required to clarify the role of 2-AG and endocannabinoid signalling in placental development. On the other hand, higher 2-AG concentrations may mimic *in vivo* abnormal levels of this eCB that may result from an abnormal expression or activity of 2-AG metabolic enzymes.

In conclusion, our data report for the first time that 2-AG interferes with the spontaneous *in vitro* functional and morphological differentiation of cytotrophoblast into syncytiotrophoblast and points to a role for this eCB during placental development, through a CB receptor-dependent mechanism. In fact, the reduction in hCG secretion, *Leptin* gene transcription and in pALP activity suggests a negative impact of 2-AG in biochemical differentiation. Furthermore, the decrease in *GCM-1* and *syncytin-2* mRNA transcripts and in the number of nuclei in syncytium indicates an impairment in cytotrophoblast fusion. We propose 2-AG as a novel player in the network of hormones, proteins and other mediators that regulate cytotrophoblast cell differentiation which are essential for a successful development of the placenta.

Author's roles

M.A.C.: experimental design, performance of all the experiments, data analysis and manuscript draft; E.K., B.M.F, N.A.T. and G.C.S.: experimental design, data analysis and manuscript draft.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

The authors thank Fundação para a Ciência e a Tecnologia (FCT) for the PhD grant of Mariana Costa (SFRH/BD/70721/2010) and for the Post-doctoral grant of Bruno Fonseca (SFRH/BPD/72958/2010). The authors also thank to the Department of Obstetrics and Gynaecology of S. João Hospital Center.

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Manuscript IV

Transient receptor potential vanilloid 1 is expressed in human cytotrophoblasts: induction of cell apoptosis and impairment of syncytialization

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The International Journal of Biochemistry & Cell Biology, 2014.
DOI: 10.1016/j.biocel.2014.10.008.

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Transient receptor potential vanilloid 1 is expressed in human cytotrophoblasts: Induction of cell apoptosis and impairment of syncytialization

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ARTICLE INFO

Article history:
Received 28 May 2014
Received in revised form
25 September 2014
Accepted 6 October 2014
Available online xxx

Keywords:
Anandamide
Apoptosis
Capsaicin
Cytotrophoblasts
Syncytialization
TRPV1

ABSTRACT

The normal development of placenta relies essentially on a balanced proliferation, differentiation and apoptosis of cytotrophoblasts. These processes are tightly regulated by several hormones, cytokines, lipids and other molecules and anomalies in these events are associated with gestational complications. The cation channel transient receptor potential vanilloid 1 (TRPV1) is expressed in several organs and tissues and it participates in cellular events like nociception, inflammation and cell death. However, the expression and importance of this receptor in human placenta still remains unknown. In this work, we found that TRPV1 is expressed in human cytotrophoblasts and syncytiotrophoblasts. Furthermore, the TRPV1 agonists capsaicin and anandamide decreased cytotrophoblast viability and induced morphological alterations, such as chromatin condensation and fragmentation, which suggest the occurrence of apoptosis. Also, either TRPV1 agonists induced a loss of mitochondrial membrane potential and an increase of caspase 3/7 activity and production of reactive species of oxygen and nitrogen. Furthermore, capsaicin (10 μM) impaired the spontaneous *in vitro* differentiation of cytotrophoblasts into syncytiotrophoblasts by triggering TRPV1, as observed by the decrease in placental alkaline phosphatase activity and in human chorionic gonadotropin secretion. On the other hand, anandamide decreased placental alkaline phosphatase activity via a TRPV1-independent mechanism but did not influence the secretion of human chorionic gonadotropin. In conclusion, we showed that TRPV1 is expressed in human cytotrophoblasts and syncytiotrophoblasts and also reported the involvement of this receptor in cytotrophoblast apoptosis and differentiation.

1. Introduction

The transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel with six- transmembrane-domain that was identified for the first time in 1997, in sensory neurons (Caterina et al., 1997). TRPV1 is sensitive to several stimuli such as noxious, heat, low pH and several endogenous and exogenous molecules (Vriens et al., 2009). It is mainly involved in temperature sensing and nociception, but TRPV1 also participates in other cellular processes such as apoptosis (Contassot et al., 2004; Pan et al., 2013; Song et al., 2013), muscle contraction (Charrua et al., 2007; Matsumoto et al., 2009; Shimizu et al., 2007), autophagy (Farfariello et al., 2012; Li et al., 2014) and inflammation (Fernandes et al., 2012; Trevisani et al., 2004; Vigna et al., 2011). Some of the endogenous ligands of TRPV1, the endovanilloids, are the endocannabinoids anandamide (AEA) and *N*-arachidonoyldopamine (NADA), the oleoylethanolamide (OEA), protons, prostaglandins and lipoxygenase (LOX) or cytochrome P450 (CYP450) products (Vriens et al., 2009). TRPV1 is present in several organs and tissues, though its expression in human placenta has not been reported yet.

Placenta is an exclusive organ of pregnancy that supports, feeds and protects the foetus. Its formation requires a proper proliferation, differentiation and apoptosis of its main cell type, the trophoblast. Trophoblast cells are subdivided in four types: (i) the villous cytotrophoblasts (CTs), which proliferate and differentiate

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http://dx.doi.org/10.1016/j.biocel.2014.10.008
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into (ii) syncytiotrophoblasts (STs), multinucleated cells involved in biosynthesis of several hormones and proteins elemental for the progression of gestation; (iii) extravillous trophoblasts (EVTs), which invade the maternal spiral artery and uterine tissues; and iv) giant trophoblast cells. Although there is no information about the expression and function of TRPV1 in human placenta, TRPV1 has already been described in rat placenta (Cella et al., 2008; Fonseca et al., 2012), where it mediates AEA-induced increase in nitric oxide synthase activity (Cella et al., 2008). Nonetheless, other types of vanilloid receptors (TRPV5 and TRPV6) were already described in human placenta (Bernucci et al., 2006; Moreau et al., 2002) and, apparently, they are important for the calcium uptake by the syncytiotrophoblast. Calcium is an important second messenger in the cytotrophoblast differentiation and EVTs migration and changes in its homeostasis are associated with preeclampasia and intrauterine growth restriction (IUGR) (Baczyk et al., 2011). Although TRPV1 is not a selective cation channel, it has preference for calcium ions, so it may be relevant for calcium signalling in cytotrophoblasts. Moreover, since TRPV1 also mediates apoptosis, it may also participate in cytotrophoblast turnover. A coordinated proliferation, differentiation and apoptosis of cytotrophoblasts is essential for the placental development and alterations in these processes are associated with pregnancy disorders (Crocker et al., 2003; Roje et al., 2011; Smith et al., 1997).

Here, we investigated the expression of TRPV1 in human cytotrophoblasts and syncytiotrophoblasts of normal term placentas. Also, we studied the influence of the TRPV1 exogenous agonist capsaicin (CPS), a component of hot chilli pepper, and of the endovanilloid AEA in human primary cytotrophoblasts apoptosis and differentiation into syncytiotrophoblasts, to study the role of this receptor in cytotrophoblast cells turnover.

2. Material and methods

2.1. Primary cultures of human cytotrophoblasts

All the procedures performed with human placentas were conducted in accordance with the Ethical Committee of Hospital S. João, Porto. Term placentas of normal pregnancies were immediately collected after delivery. Then, human cytrotrophoblast cells (hCTs) were isolated, as previously described (Keating et al., 2007). Briefly, villous tissue was dissected and digested in a trypsin and DNAse I-containing solution and cells were separated in a discontinuous percoll gradient (GE Healthcare, Buckinghamshire, UK). Cytotrophoblasts were collected and seeded in 96 and 24-well plates, 21 cm^2 dishes or 8-well chamber slides, at densities 1.35×10^5, 1×10^6, 1×10^7 or 6×10^5, respectively, in DMEM/F12 medium (Sigma–Aldrich Co., St. Louis, MO, USA) supplemented with 10% (v/v) of FBS and an antibiotic–antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericine B) (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and were incubated at 37 °C and 95% air/5% CO2 humidified atmosphere. After 12 h of adhesion, for the cell death related-assays, cell culture medium was replaced by fresh medium supplemented with 1% FBS. For differentiation studies, cells were kept in medium supplemented with 10% FBS, since it is an experimental requirement for the spontaneous *in vitro* differentiation of cytotrophoblasts into syncytiotrophoblasts.

2.2. Expression of TRPV1 at 12 and 72 h of culture

Human cytotrophoblasts and syncytiotrophoblasts (at 12 and 72 h of culture, respectively) were collected from 21 cm^2 plates in TRIzol® reagent (Gibco/Invitrogen Corporation, Carlsbad, CA, USA). Total RNA and protein was extracted according to manufacturer's instructions. RNA was quantified in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and its quality assessed with the Experion RNA StdSens Kit and analysed with the Experion analytical software (Bio-Rad Laboratories, USA). RNA was reverse transcribed into cDNA with the iScript™ Select cDNA Synthesis (Bio-Rad Laboratories, USA). For the analysis of *TRPV1* gene transcription, cDNA was amplified with KAPA SYBR® FAST qPCR Master Mix 2x Kit (Kapa Biosystems, Woburn, MA, USA), according to the kit protocol, in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Specific primers for human TRPV1 amplification were used (sense 5′-CAAGAACATCTGGAAGCTGC-3′; antisense 5′-CTTCTCCCCGGAAGCGGCAGG-3′ (Ludanyi et al., 2008)) and the annealing temperature was set at 62 °C. The specificity of the amplification PCR product was evaluated by the melting curve analysis.

For Western Blot, protein samples were loaded in a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). After blocking the nonspecific binding sites with a blocking solution (5% dry milk in PBS with Triton™ X-100 0. 1%), membranes were incubated with anti-TRPV1 (goat; 1:100; Santa Cruz Biotechnology, Dallas, Texas, USA; sc-12500) overnight, at 4 °C. Then, they were incubated with peroxidase-conjugated secondary antibody (rabbit anti-goat, 1:1000; Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 h, at room temperature. Lastly, membranes were exposed to WesternBright™ ECL (Advansta, Menlo Park, USA) and then to x-ray film (Kodak XAR; Eastman Kodak, Rochester, NY). Membranes were stripped and re-incubated with anti-β-tubulin antibody (rabbit; 1:500; Santa Cruz Biotechnology, Dallas, Texas, USA), for loading control. Rat brain was used as positive control. The signal intensity of the bands was analysed by densitometry (BIO-PROFIL Bio-1D2; Vilber Lourmat, Marne-la-Vallée, France) and normalized for the corresponding β-tubulin band; the results were expressed in arbitrary units.

For immunocytochemistry studies, cells at 12 and 72 h of culture seeded in 8-well chamber slides were fixed with a 4% paraformaldeylde solution. For immunohistochemistry, deparaffined slides of human placenta (4 μm thick) were used. The expression of TRPV1 was analysed by an avidin–biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). The non-specific binding sites were blocked and slides were incubated with anti-TRPV1 antibody (1:100) overnight at 4 °C. Slides were then incubated with biotinylated secondary antibody followed by incubation with Vectastain ABC-AP reagent, according to the manufacturer's instructions. The reaction was developed with Sigma Fast Red™ tablets (Sigma–Aldrich Co., St. Louis, MO, USA), slides were counterstained with Mayer's hematoxylin solution (Sigma–Aldrich Co., St. Louis, MO, USA) and mounted in Aquamount medium (BDH Laboratory Supplies, Poole, England). Negative control was performed by the replacement of the primary antibody by goat IgG.

2.3. Cell viability assays

For cell viability assessment, MTT assay was used. hCTs were plated in 96-well plates and, after adhesion, cells were incubated with or without capsaicin (Tocris Bioscience, Bristol, UK) (1-25 μM) for 12 and 24 h. The involvement of TRPV1 in the effects induced by CPS and anandamide (AEA; Tocris Bioscience, Bristol, UK) was evaluated after a preincubation with 200 nM Capsazepine (CPZ) or 20 nM 5'-Iodoresiniferatoxin (5-iRTX) (Tocris Bioscience, Bristol, UK), two TRPV1 antagonists, 30 min before the addition of CPS or AEA (25 or 15 μM, respectively). At the end of incubation period, the yellow tetrazole MTT (0. 5 mg/ml final concentration) was added to the cells for 2 h 30 min, at 37 °C. The purple formazan formed was extracted by a solution of Dimethylsulfoxide (DMSO):isopropanol

(3:1) and quantified by spectrophotometry, at 540 nm, in a Multiscan Ascent microplate reader.

2.4. Morphological studies

hCTs seeded in 8-well chamber slides were treated with CPS (25 μM) for 24 h and fixed with a 4% paraformaldehyde solution. Giemsa stain was added to the cells for 30 min and then, cells were observed by light microscopy. For Höechst staining, cells were incubated with 0.5 mg/ml Höechst 33342 for 20 min and observed by fluorescence microscopy equipped with an excitation filter with maximum transmission at 360/40 nm (Eclipse Ci, Nikon, Japan).

For the transmission electron microscopy (TEM), cells were harvested by trypsinization and fixed with a solution of 2% glutaraldehyde/4% p-formaldehyde. The cells were post-fixed with 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812 and ultra-thin sections (40–60 nm thickness) were cut on a LKB 2188 NOVA Ultramicrotome (LKB NOVA, Bromma, Sweden), using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 15 min each and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100 W Tokyo, Japan at the HEMS - Institute for Molecular and Cell Biology (IBMC) of the University of Porto.

2.5. Determination of Caspase 3/7 activity

hCTs were plated in 96-well white plates and treated with AEA (15 μM) or CPS (25 μM) for 20 h, in the absence or presence of CPZ. At the end of incubation period, Caspase-Glo® 3/7 reagent was added, according to manufacturer's instructions. Cells were incubated for 30 min, at room temperature, and the luminescence was assessed in a 96-well Microplate Luminometer (BioTek Instruments, Vermont, USA). The positive control was performed with the incubation of cells with 100 nM of staurosporine (STS; Sigma–Aldrich Co., St. Louis, MO, USA). The results were expressed in relative light units (RLU).

2.6. Assessment of mitochondrial membrane potential ($\Delta\psi m$) and quantification of intracellular reactive oxygen and nitrogen species (ROS/RNS)

hCTs were seeded in 96-well black plates and treated with AEA (15 μM) or CPS (25 μM), in the absence or presence of CPZ. For $\Delta\psi m$, cells treated for 20 h were washed and incubated with $DiOC_6$ 100 nM (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) for 20 min, at 37 °C, in the dark. For ROS/RNS assessment, cells were incubated with the fluorescent probe 2, 7-Dichlorodihydrofluorescein diacetate (DCDHF-DA; Sigma-Aldrich Co., St. Louis, MO, USA) for 1 h, at 37 °C. After pre-treatment with TRPV1 antagonists, AEA or CPS were added for 1 h. The fluorescence was measured in a Microplate Fluorimeter (BioTek Instruments, Vermont, USA) (excitation-485+/−10 nm; emission 530+/−12.5 nm). The mitochondrial membrane depolarizing agent carbonyl cyanide m-chlorophenylhydrazone (CCCP; 10 μM) or H_2O_2 (200 μM) (Sigma–Aldrich Co., St. Louis, MO, USA) were used as positive control for $\Delta\psi m$ and ROS/RNS, respectively. The results were expressed as percentage of control, comparing loss of mitochondrial membrane potential or the generation of ROS/RNS induced by CPS or AEA with untreated cells.

2.7. Ecto-placental alkaline phosphatase (pALP) activity assay

After adhesion, hCTs plated in 24-well plates were treated with CPS and AEA (1-20 μM), for 48 h. To investigate the involvement of TRPV1, cells were treated with CPS and AEA (10 μM), in the absence or presence of CPZ or 5-iRTX. After incubation, the assay of ecto-pALP activity was performed as described by Keating et al. (Keating et al., 2009). Briefly, cells were washed and incubated with the pALP substrate *p*-nitrophenilphosphate (2.5 mM; Sigma Chemical Co, St. Louis, MO, USA), in the presence of $MgCl_2$ (100 mM; Sigma Chemical Co, St. Louis, MO, USA), at 37 °C for 1 h. The reaction was stopped by the addition of an ice-cold solution of 0.02 M NaOH and the absorbance was quantified at 405 nm, in a Multiscan Ascent microplate reader. The *p*-nitrophenol (pNP) formed was determined by interpolation in a calibration curve of pNP (Sigma Chemical Co, St. Louis, MO, USA). Protein amount was assessed by Bradford assay, after cell lysis with Triton X-100 0.1% in Tris-HCl 5 mM (pH 7.4). The incubation with L-Phenylalanine (2 mM; Sigma Chemical Co, St. Louis, MO, USA), a pALP inhibitor, for 30 minutes before the addition of pALP substrate was used as a control. The results were expressed as nmol of *p*-nitrophenol/mg of protein/minute, in relative values, in comparison to the control.

Equimolar concentrations of the vehicle (ethanol 0.1%) had no effects on any of the parameter studied in the cytotrophoblast cells (data not shown).

2.8. Quantification of secreted β-hCG

The supernatant of cells treated in the conditions referred for the ecto-pALP assay was collected, centrifuged and stored at −80 °C. The effects of AEA and CPS in hCG production was evaluated by the quantification of its β subunit in cell supernatants by ELFA (Enzyme Linked Fluorescent Assay), by using the VIDAS HCG kit (bioMérieux SA, Marcy l'Etoile, France) in the mini-VIDAS autoanalyser (bioMérieux SA, Marcy l'Etoile, France), according to manufacturer's protocol. The results were normalized for the total protein content, calculated as mIU/mg of protein and expressed as relative values, in comparison to the control.

2.9. Measurement of intracellular calcium levels

hCTs plated in 96-well black plates were incubated with the calcium probe 5 μM Fluo-3 AM (Molecular Probes, Eugene, OR, USA) diluted in FBS-free culture media, at room temperature, in the dark. Then, cells were washed and incubated without the probe, at 37 °C, to allow complete de-esterification of intracelluar Fluo-3 AM esters. Cells were treated with AEA (15 μM) or CPS (25 μM), in the absence or presence of CPZ and the fluorescence was immediately registered in a Microplate Fluorimeter (BioTek Instruments, Vermont, USA) (excitation-485+/−10 nm; emission 530+/−12.5 nm), at 37 °C, from 10 to 10 s, during 2 min and 30 s. To corroborate that $[Ca^{2+}]_i$ alterations result from Ca^{2+} influx from the extracellular environment, ethylene glycol tetraacetic acid (EGTA; 1 mM) was added to the cells prior to the incubation with AEA and CPS (data not shown). Intracellular calcium levels where calculating according to the formula $[Ca^{2+}]_i = Kd\ [F - F_{min}]/[F_{max} - F]$, where the Kd corresponds to the dissociation constant of the dye for Ca^{2+} (400 nM). F_{max} was considered the fluorescence of the calcium ionophore A23187 (5 μM) and the F_{min} the fluorescence in the absence of calcium, obtained after adding 10 mM EGTA to the A23187-treated cells.

2.10. Statistical analysis

Statistical analysis was carried out by one or two-way ANOVA, followed by the Bonferroni post-hoc test to make pairwise comparisons of individual means (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). The results correspond to the

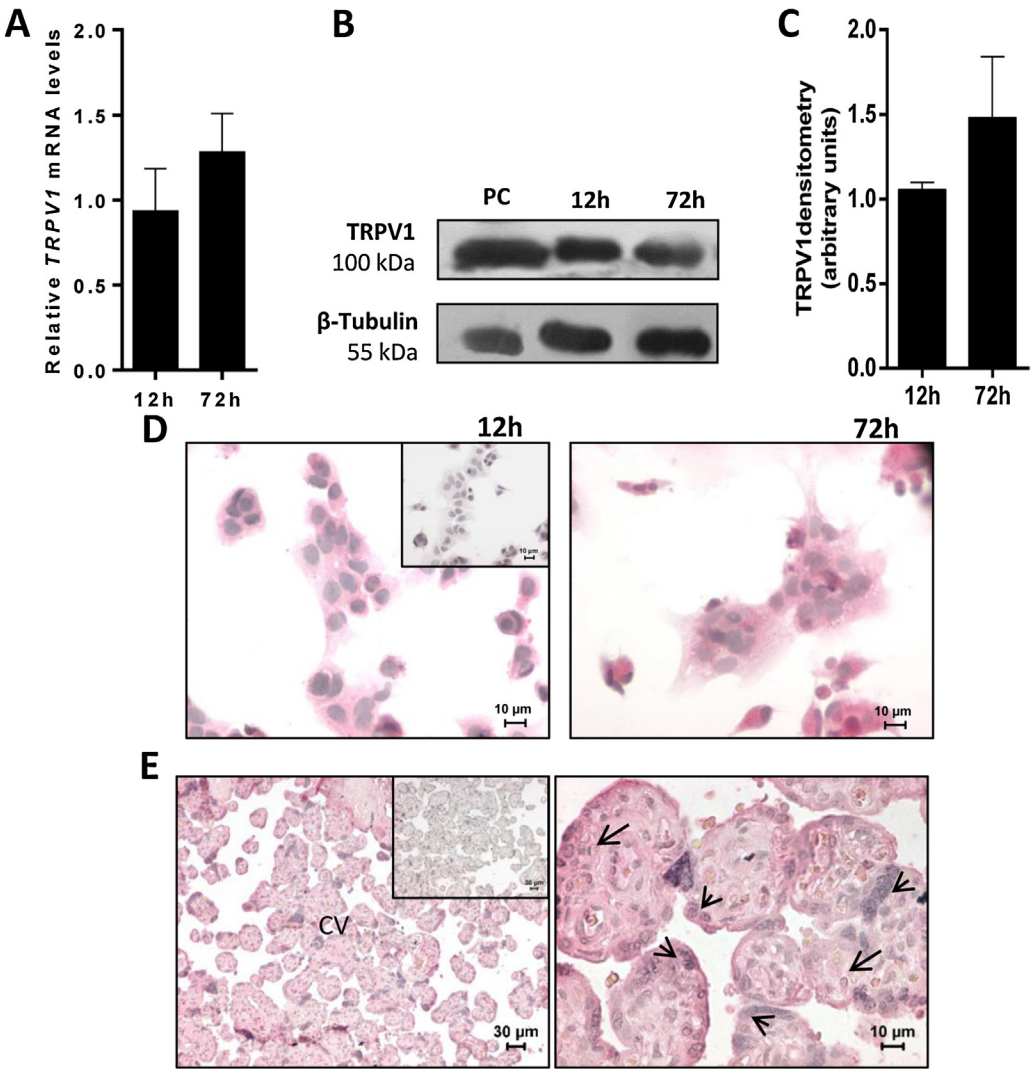

Fig. 1. Expression of the Transient Receptor Potential Vanilloid 1 (TRPV1) in human cytotrophoblasts (hCTs) and syncytiotrophoblasts (hSTs), corresponding to 12 and 72 h of culture, respectively. (A) RT-PCR revealed that *TRPV1* is transcribed in both hCTs and hSTs but there are no significant differences in its transcription in these two cell types. (B) Representative western blot showed that this receptor is expressed in hCTs and hSTs; rat brain was used as positive control (PC) and β-tubulin was used as a loading control. (C) Densitometry analysis revealed that TRPV1 is expressed in similar levels, at 12 and 72 h. Immunocytochemistry (D) and immunohistochemistry (E) demonstrated TRPV1 expression in both hCTs and hSTs; arrows and arrowheads indicate the hCTs and hST, respectively. The squares inserted in D and E show a negative control. CV-chorionic villi.

mean of at least three independent experiments performed in triplicate. The results were expressed as mean ± SEM and differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Expression of the transient receptor potential vanilloid 1 (TRPV1) in cytotrophoblasts and syncytiotrophoblasts

We investigated the presence of TRPV1 in human trophoblasts, at 12 and 72 h of culture, corresponding to CTs and STs, respectively. *TRPV1* gene amplification by RT-PCR revealed similar transcript levels in CTs and STs (Fig. 1A). Western Blot densitometry corroborated RT-PCR data, showing that TRPV1 is expressed in CTs and STs with no significant differences (Figs. 1B, C). Immunocytochemistry (Fig. 1D) and immunohistochemistry (Fig. 1E) demonstrated that this receptor is expressed in both CTs and STs.

3.2. Effects of the TRPV1 agonists capsaicin and anandamide in cytotrophoblast cell viability and morphology

As TRPV1 was expressed in hCTs, we intended to study its function in these cells. The TRPV1 agonist Capsaicin (CPS) induced a decrease in cell viability, for doses of 10 μM and above (Fig. 2A). These effects on cell viability were not accompanied by LDH release (Fig. 2B). Cells treated with CPS (25 μM) presented chromatin condensation as observed by Giemsa (Fig. 3A and B) and Höechst assays (Fig. 3C and D). Transmission electron microscopy showed chromatin condensation, drastic membrane alterations with loss of typical cytotrophoblast microvilli and membrane blebbing (Fig. 3E and F).

To investigate the involvement of TRPV1 in the decrease of cell viability induced either by CPS (25 μM) or AEA (15 μM), hCTs were preincubated with CPZ and 5-iRTX, two antagonists of this receptor. Both antagonists were able to partially reverse the effects of CPS and of AEA (Fig. 4).

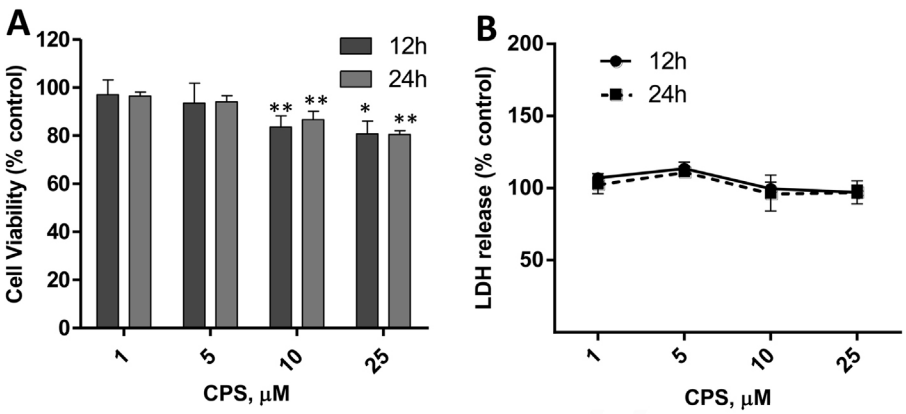

Fig. 2. Effects of the TRPV1 agonist, capsaicin (CPS), in human cytotrophoblast cells viability. (A) After 12 and 24 h treatments, CPS induced a decrease in cell viability, for concentrations higher than 10 μM but without LDH release (B). (**$p < 0.01$, *$p < 0.05$ vs. Control).

3.3. *Effects of capsaicin and anandamide in caspase 3/7 activity, mitochondrial membrane potential (Δψm) and generation of reactive species of oxygen and nitrogen (ROS/RNS)*

To study if CPS and AEA effects in cytotrophoblast cells viability resulted from the activation of an apoptotic process, we evaluated the impact of these compounds in Δψm and caspase 3/7 activity. CPS (25 μM) induced a 20%-loss of Δψm, in comparison to the control (Fig. 5A), and increased caspase 3/7 activity in 23% (Fig. 5B). Both effects were reversed by the TRPV1 antagonist, CPZ. AEA induced a loss of Δψm in 15% (Fig. 5A) and increased caspase 3/7 activity in 28% (Fig. 5B). These AEA effects were both attenuated by CPZ. Furthermore, CPS (25 μM) increased ROS/RNS production in 16%, while AEA (15 μM) induced an increase of 43%, in comparison to control (Fig. 5C). Once again, these effects were mediated by TRPV1, since CPZ was able to significantly reverse the ROS/RNS generation.

3.4. *Influence of TRPV1 in spontaneous differentiation of human cytotrophoblasts into syncytiotrophoblasts*

To study the involvement of TRPV1 in the differentiation process of hCTs into syncytiotrophoblasts, we investigated the impact of CPS and AEA (10 μM) in two biochemical differentiation markers, pALP activity and hCG secretion, after a 48 h treatment. The concentration of 10 μM was chosen because it was the lowest concentration that affected the pALP activity (data not shown). We observed that CPS reduced pALP activity and hCG secretion in 29% and 33%, respectively (Fig. 6A and B); these effects were partially reversed by both TRPV1 antagonists, CPZ and 5-iRTX. On the other

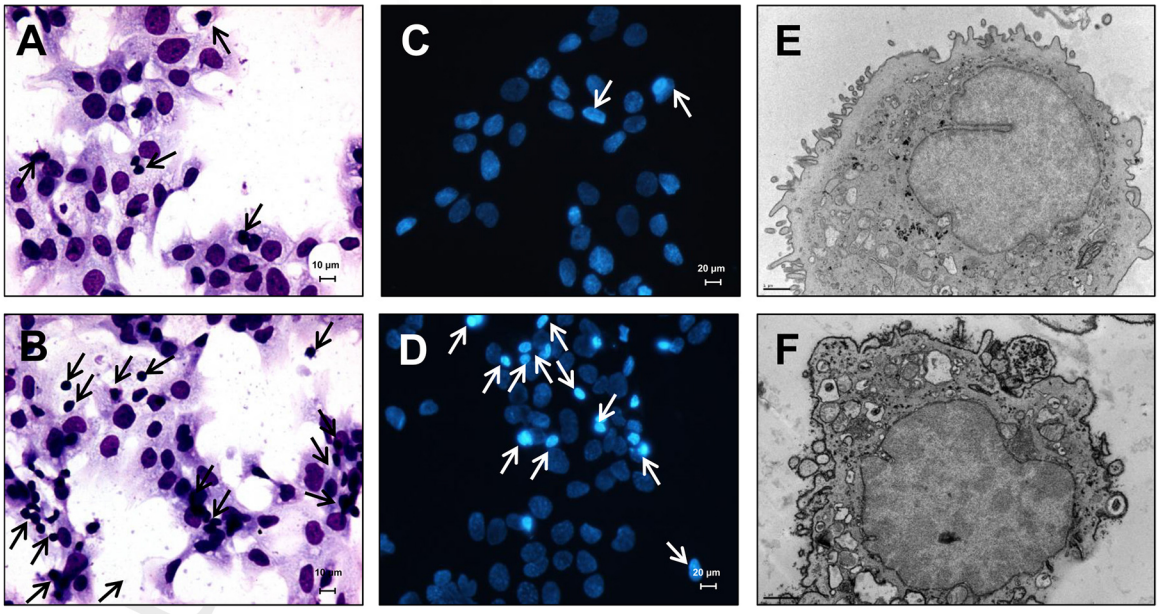

Fig. 3. Effects of capsaicin (CPS) in human cytotrophoblast cells morphology. After a 24 h treatment, Giemsa staining showed chromatin condensation (arrows) (B), in comparison to untreated cells (A). (D) Höechst also supported the presence chromatin condensation (arrows) in CPS treated cells (D), when compared to the control (C). (E) Transmission electron microscopy (TEM) showing the structural features of the cytotrophoblasts. Cell surface exhibits a typical microvillous membrane, the cytoplasm contains the usual organelles and a dispersed chromatin in the nucleus. (F) In contrast, CPS-treated cells presented drastic loss of microvilli, membrane blebbing and chromatin condensation.

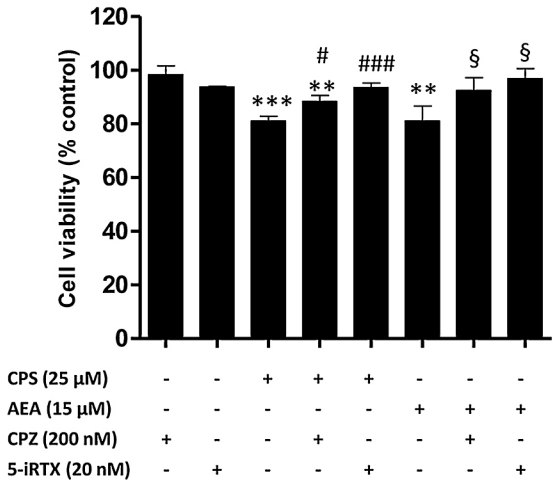

Fig. 4. Involvement of TRPV1 in capsaicin (CPS) and anandamide (AEA)-induced viability loss of human cytotrophoblasts (hCTs), after a 24 h treatment. CPS (25 μM) decreased hCTs viability in 20% and both TRPV1 antagonists capsazepine (CPZ) and 5′-Iodoresiniferatoxin (5-iRTX) partially reversed this effect. Moreover, the effect on cell viability induced by the endovanilloid AEA was also reversed by both antagonists. (***$p < 0.001$, **$p < 0.01$ vs. Control; ###$p < 0.001$, #$p < 0.05$ vs. CPS 25 μM; §$p < 0.05$ vs. AEA 15 μM). TRPV1-Transient Receptor Potential Vanilloid 1.

hand, AEA decreased pALP activity in 27% but did not interfere with hCG secretion (Fig. 6A and B). Moreover, the effect on pALP activity was TRPV1-independent.

3.5. Influence of TRPV1 in the intracellular calcium levels ($[Ca2+]_i$)

Since TRPV1 is a cation channel with preference for calcium ions and its activation mediates an increase in intracellular calcium, we studied the effects of TRPV1 agonists in the intracellular levels of this cation. We concluded that both CPS (25 μM) and AEA (15 μM) induced a rapid increased of $[Ca^{2+}]_i$ reaching peak increases of 3 and 1,8 fold, respectively, after 20 s of exposure. These effects were reversed by CPZ, suggesting that this Ca^{2+} influx occurs through TRPV1 (Fig. 7). The cell incubation with EGTA (1 mM) abolished the CPS and AEA-induced increase in intracellular Ca^{2+} levels (data not shown), corroborating that this increase results from a Ca^{2+} influx from extracellular medium.

4. Discussion

A successful pregnancy outcome depends on a proper development of the placenta. This process is highly coordinated and

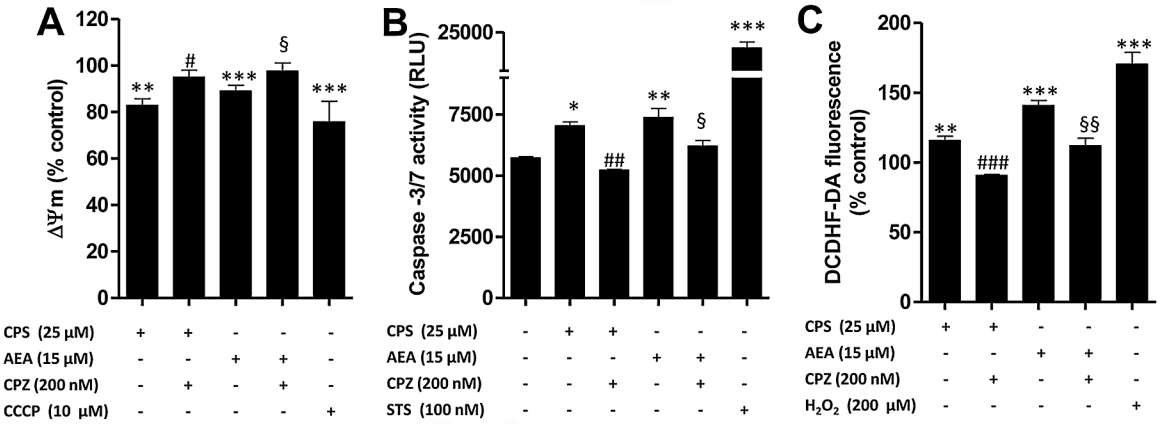

Fig. 5. Impact of capsaicin (CPS) and anandamide (AEA) in mitochondrial membrane potential (Δψm), caspase 3/7 activity and production of reactive species of oxygen and nitrogen (ROS/RNS), in human cytotrophoblasts (hCTs). (A) After 20 h of treatment, CPS (25 μM) and AEA (15 μM) induced a loss of Δψm and their effect was reversed by the TRPV1 antagonist capsazepine (CPZ). (B) Both CPS and AEA increased caspase 3/7 activity and CPZ was also able to reduce this increment. (C) After 1 h of treatment, CPS and AEA increased the levels of ROS/RNS, effect that was reversed by the TRPV1 antagonist. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. Control; ###$p < 0.001$, ##$p < 0.01$, #$p < 0.05$ vs. CPS 25 μM; §§$p < 0.01$, §$p < 0.05$ vs AEA 15 μM).

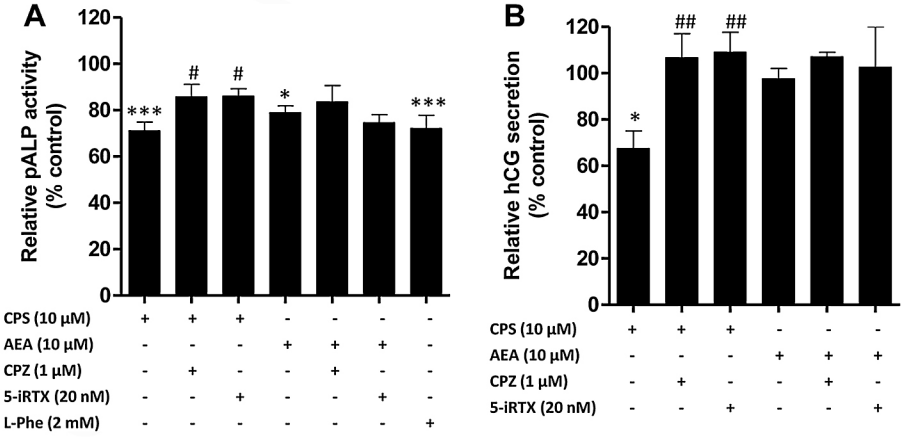

Fig. 6. Influence of the TRPV1 agonists capsaicin (CPS) and anandamide (AEA) in the differentiation of human cytotrophoblasts into syncytiotrophoblasts, after a 48 h treatment. (A) CPS and AEA (10 μM) decreased pALP activity but, whereas CPS effect was reversed by the TRPV1 antagonists capsazepine (CPZ) and 5′-Iodoresiniferatoxin (5-iRTX), AEA effect was not dependent on TRPV1 activation. (B) CPS reduced hCG secretion but AEA did not significantly alter the production of this hormone; CPS effect was also reversed by CPZ and 5-iRTX. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. Control; ##$p < 0.01$, #$p < 0.05$ vs CPS 10 μM).

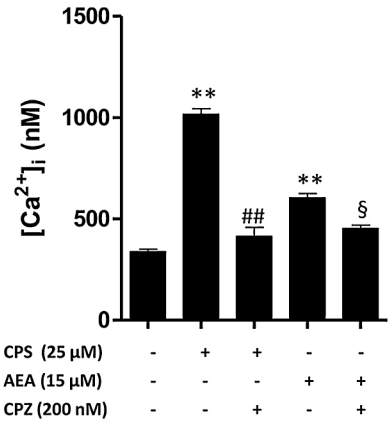

Fig. 7. Influence of TRPV1 activation by capsaicin (CPS) and anandamide (AEA) in the intracellular calcium levels ($[Ca^{2+}]_i$). Both CPS (25 μM) and AEA (15 μM) induced a rapid increase of $[Ca^{2+}]_i$, effect that was reversed by the TRPV1 antagonist capsazepine (CPZ). (**$p < 0.01$ vs. Control; ##$p < 0.01$ vs. CPS 25 μM; §$p < 0.05$ vs AEA 15 μM).

involves proliferation, differentiation and death of trophoblasts, the specialized epithelial cells of the placenta. These cells produce hormones, lipids, cytokines and other molecules that integrate a complex network essential for the homeostasis of the foeto-placental microenvironment. The cation channel TRPV1 was initially identified in sensory neurons (Caterina et al., 1997) but, more recently, its expression in other cells has been described. Although TRPV1 has already been identified in rat placenta (Cella et al., 2008; Fonseca et al., 2012), its presence and function in the human placenta has not been investigated yet. In this work, we reported that cytotrophoblasts and syncytiotrophoblasts express TRPV1. In addition, we showed that the activation of this receptor induces apoptosis in cytotrophoblasts and impairs their differentiation into syncytiotrophoblasts.

In spite of the TRPV1 expression in human trophoblasts was unknown till now, other vanilloid receptors (TRPV5 and 6) were already identified in these cells, where they seem to regulate calcium transport (Bernucci et al., 2006; Moreau et al., 2002). Moreover, it was described that their expression was decreased in preeclamptic placentas, impairing the Ca^{2+} transport in the syncytiotrophoblast (Hache et al., 2011). TRPV1 is a non-selective cation channel but it has preference for calcium ions. According to our results, TRPV1 modulates the intracellular levels of this cation in cytotrophoblasts, contributing to the calcium homeostasis in these cells. In addition, since calcium is important for cell differentiation and apoptosis (Baczyk et al., 2011) and our results indicate that TRPV1 activation interfere with these processes in hCTs, the endovanilloid signalling may be relevant for the regulation of the cytotrophoblast cells turnover.

We showed that TRPV1 exogenous ligand CPS decreased cell viability for concentrations of 10 μM or above and induced chromatin condensation and fragmentation. Moreover, CPS and the endovanilloid AEA were able to increase caspase 3/7 activity, to induce loss of $\Delta\psi m$ and to increase ROS/RNS generation. All these effects were significantly attenuated by the TRPV1 antagonist, CPZ. These data indicate that TRPV1 activation in hCTs induces apoptosis, by a mechanism that involves the intrinsic pathway. In agreement with our results, it was already shown that CPS increases ROS production in synoviocytes, neurons and thymocytes (Amantini et al., 2004; Hu et al., 2012; Shirakawa et al., 2008) and induces loss of $\Delta\psi m$ in glioma and human KB cancer cells (Amantini et al., 2007; Lin et al., 2013). Nevertheless, it was also reported that TRPV1 activation induces alterations in other interveners of the intrinsic pathway, such as caspase 9 activation or cytochrome c release in thymocytes and cancer cells (Amantini et al., 2009; Amantini et al., 2004; Lin et al., 2013; Skrzypski et al., 2014). Furthermore, CPS-induced apoptosis may also alter Ca^{2+} influx (Amantini et al., 2004; Hu et al., 2012; Shirakawa et al., 2008) and phosphorylation of mitogen-activated protein kinases (MAPK) p38 (Amantini et al., 2007; Yamaji et al., 2003), p42/p44 (Shirakawa et al., 2008) or JNK (Yamaji et al., 2003). The endovanilloid AEA also induces apoptosis in different cell types via TRPV1-dependent stimulation (Contassot et al., 2004; Maccarrone et al., 2000). In human endothelial cells, AEA induced apoptosis via TRPV1, by inducing the phosphorylation of MAPKs p38 and JNK and caspase 3 activation (Yamaji et al., 2003). These diverse pathways suggest that the apoptotic signalling triggered by TRPV1 is cell specific.

A thin balance between cytotrophoblast proliferation and apoptosis is crucial for placentation. A deregulation of these processes has been associated with gestational complications such as preeclampsia or IUGR (Crocker et al., 2003; Leung et al., 2001; Longtine et al., 2012; Roje et al., 2011; Smith et al., 1997; Tomas et al., 2011). Moreover, a deregulation in oxidative and nitrative stress and antioxidant defences is also correlated with these pathologies (Coughlan et al., 2004; Mert et al., 2012; Sharma et al., 2006). Since TRPV1 activation induces both apoptosis and ROS/RNS generation, a tight control of endovanilloid signalling may be required for a successful pregnancy outcome. Moreover, AEA is an endogenous lipid that is physiologically present in the human placenta (Marczylo et al., 2010) and here we report that it induces hCT apoptosis through TRPV1, suggesting a role for the endovanilloid signalling in this cellular event.

Besides apoptosis, CPS also impaired the biochemical differentiation of human cytotrophoblasts into syncytiotrophoblasts, as observed by the decrease in the differentiation markers pALP activity and hCG secretion. Both effects were reversed by CPZ and 5-iRTX, indicating that TRPV1 may be involved in the modulation of cytotrophoblast differentiation. Recently, a role for TRPV1 in cell differentiation has emerged. Indeed, the activation of this receptor by CPS inhibited the cytokine-stimulated differentiation of immature dendritic cells (Toth et al., 2009). On the other hand, the inhibition of TRPV1 by CPZ impaired osteoblasts and osteoclasts differentiation (Idris et al., 2010), suggesting that TRPV1 signalling is important for the differentiation of these bone cell types. Moreover, TRPV1 activation is important for the TGF-β1-induced formation of myofibroblasts from corneal fibroblasts (Yang et al., 2013). For a normal course of pregnancy, it is required a controlled hCTs differentiation and deregulations in this process are associated with gestational complications (Langbein et al., 2008; Lim et al., 1997; Ruebner et al., 2010). Since TRPV1 activation seems to interfere with this process, it is suggested that the endovanniloid signalling may be important for its modulation.

Contrary to CPS, the endovanniloid AEA decreased pALP activity via a TRPV1-independent mechanism and had no significant effects on hCG secretion, which is in agreement with a previous work (Williams et al., 2008). These data suggest that AEA may not impair hCT differentiation. One possible explanation for these results is the agonist activity of AEA on cannabinoid receptors CB1 and CB2 and Peroxisome proliferator-activated receptor γ (PPAR-γ) (Fonseca et al., 2013), which are also expressed in human trophoblasts (Habayeb et al., 2008; Schaiff et al., 2000). We hypothesize that AEA may simultaneous activate these receptors triggering different cellular pathways and, consequently, masking its effects on TRPV1. Nevertheless, the CPS-induced impairment in cell differentiation showed that TRPV1 activation may interfere with syncytialization and so, it is possible that other endovanilloids such as OEA, prostaglandins or LOX and CYP450 metabolites, may have a physiological role in this cellular event.

In conclusion, we reported for the first time the expression of TRPV1 in human cytotrophoblasts and syncytiotrophoblasts.

Moreover, TRPV1 activation by CPS and AEA mediated the apoptotic process in hCTs by activation of the intrinsic pathway and CPS also impaired syncytialization. In this way, our data indicate that the endovanilloid signalling pathway may be a novel player in the network of molecules that modulate cytotrophoblasts turnover.

Conflict of interests

The authors declare that they have no competing interests.

Acknowledgments

The authors thank Fundação para a Ciência e Tecnologia (FCT) for the PhD grant of Mariana Costa (SFRH/BD/70721/2010) and for the Post-doctoral grant of Bruno Fonseca (SFRH/BPD/72958/2010). The authors also thank to Department of Obstetrics and Gynaecology of S. João Hospital Center, Porto, Portugal.

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PART III

Discussion and general conclusions

In the last years, ECS research has unveiled new players in several physiological and pathophysiological processes, such as apoptosis, inflammation, neuromodulation or reproduction. Endocannabinoids have been described as important modulators in embryo transport and implantation, decidualization and labour [140, 141, 410]. Furthermore, an abnormal endocannabinoid signalling has been associated with an impairment of these reproductive events, and concomitantly, with disorders like infertility and miscarriages [410]. A proper placental development is crucial for the success of pregnancy and involves a balanced proliferation, differentiation and apoptosis of the main foeto-placental cells, the trophoblasts [85]. Since these cellular events are tightly regulated by a complex network of hormones, cytokines and other molecules, it was postulated that the eCBs might also have a role during placentation. Although information about eCBs function during placental development is lacking, the presence of AEA metabolic enzymes, NAPE-PLD and FAAH, and CB receptors were already described in human placenta [139, 392, 401]. It was also reported that AEA decreases BeWo cells viability, through a CB2 receptor-dependent mechanism [139]. In contrast, the effects and role of the other major endocannabinoid 2-AG in reproductive events have been less explored, especially during placentation.

In this study, 2-AG main biosynthetic and hydrolysing enzymes, DAGL-α and MAGL, respectively, were found to be expressed in primary human cytotrophoblasts and syncytiotrophoblasts and in the cytotrophoblast cell model BeWo cells. The expression of the cation channel TRPV1, an AEA receptor, was also demonstrated. There was no differences between CTs and STs in *DAGL-α, MAGL* and *TRPV1* mRNA levels and protein expression, suggesting that the expression is not depend on cell differentiation stage. Thus, the human trophoblasts are provided with the enzymatic machinery required to regulate the *in situ* levels of both major eCBs, AEA and 2-AG, indicating that these lipid mediators may have a role in placentation. Moreover, TRPV1 expression in trophoblasts raises questions about the role of the endovanniloid signalling during placental development. Although this receptor was initially described in neuronal cells and identified as a modulator of nociception or temperature sensing [205], it is now known that TRPV1 is expressed in several other cell types and participates in other biological processes, such as apoptosis, muscle contraction or inflammation [211, 212, 221]. Furthermore, TRPV1 was identified in rat placenta, though its function remains unclear [394, 411]. After a deeper knowledge of the presence of ECS members in placenta, the impact of AEA and 2-AG in CT proliferation, apoptosis and differentiation into STs, as well as the involvement of CB and TRPV1 receptors in these cellular events, was evaluated.

The proliferation and apoptosis studies were mainly performed in BeWo cells, since primary human CTs do not proliferate *in vitro* and spontaneously differentiate into STs, hindering the study of the cytotrophoblast cell turnover [412]. In addition, as BeWo cells are a widely used model of CTs, proliferate and express the members of ECS, they were considered a suitable model to our studies. Both AEA and 2-AG decreased BeWo cell viability and proliferation, which is, in the case of AEA, in accordance to other study already described by Habayeb *et al.* [139]. Moreover, cells treated with eCBs present morphological alterations such as chromatin condensation and fragmentation and the presence of apoptotic bodies, which are typical characteristics of the apoptotic cell death. In addition, both eCBs induced an activation of caspases-3/7 and -9, a loss of mitochondrial membrane potential ($\Delta\psi m$) and generated reactive species of oxygen and nitrogen (ROS/RNS). This suggests the involvement of the mitochondrial pathway, which was dependent on CB receptor. In fact, both CB receptor antagonists reversed AEA and 2-AG effects on $\Delta\psi m$ and ROS/RNS production, whereas caspase activities and 2-AG viability loss were only reversed by CB2 blockade. In this way, CB2 seems to be essential to the execution of the apoptotic process, though CB1 activation was also involved in the apoptotic events.

The importance of the apoptotic process during the placental development is widely accepted, since it permits the continuous trophoblast cell turnover, by removing the quiescent cells and allowing the arising of new ones. It was recently reported that villous cytotrophoblasts undergo caspase-dependent apoptosis [86] and that both extrinsic and intrinsic apoptotic pathways participate in this cellular event [89].

The role of eCB in cell apoptosis, namely by the activation of mitochondrial pathway, has been described in various cell types. Indeed, both AEA and 2-AG induce programmed cell death in rat decidual cells through the activation of CB1 [175, 297]. Furthermore, 2-AG induces apoptosis in hepatic stellate cells by a mechanism that involves mitochondrial ROS production [178], whilst AEA also activates this process in several tumour cell types, by causing cytochrome *c* release, MAPKs phosphorylation, ceramide accumulation or increase in the intracellular Ca^{2+} levels [192, 326, 327, 413]. AEA is also able to induce apoptosis in other non-tumour cells, like pancreatic β-cells or chondrocytes [154, 414].

In this work, it was also observed that AEA increased caspase-8 activity through the activation of CB receptors and the formation of truncated Bid (tBid), which suggests activation of the death receptor pathway. The participation of this apoptotic pathway in AEA-induced cell death has been described in other cell types. In fact, AEA induced apoptosis in cholangiocarcinoma cells, by accumulation of ceramide and recruitment of

Fas and FasL into lipid rafts, through the activation of GPR55 [331, 332]. In addition, AEA mediated cell death by activation of both extrinsic and intrinsic pathways in Chang liver cells [333] and chondrocytes [154]. However and contrary to the results reported in BeWo cells, these effects were independent on CB receptors, which indicates that the mechanisms involved in AEA-induced apoptosis may be cell specific.

Caspase-8 participates in non-apoptotic cellular events like proliferation, migration and differentiation, which are mainly triggered by the activation of NF-κB [415]. On the other hand, some authors suggested that this protease may be involved in cytotrophoblast differentiation, though this point has raised some controversies [39, 77, 78]. In BeWo cells, we demonstrated that AEA treatment did not activate NF-κB, since the expression of IκB-α (NF-κB inhibitor protein) was upreregulated, supporting a role for caspase-8 in AEA-induced apoptosis. In addition, AEA treatment increased the formation of truncated Bid (tBid), which results from Bid cleavage by caspase-8 [416]. Thus, these results suggest that AEA may mediate a crosstalk between the two apoptotic pathways in BeWo cells. Contrary to AEA, preliminary results indicate that 2-AG did not alter the activity of this protease in BeWo cells and that the pathways triggered in these cells by the two major eCBs may differ.

To further study the effects of eCBs in cytotrophoblast cells death, the apoptotic process was also investigated in primary human CTs. It was verified that AEA induce apoptosis in these cells, as indicated by the presence of apoptotic morphological features, increase in caspase-3/7 activity, loss of $\Delta\psi m$, cleavage of poly (ADP-ribose) polymerase 1 (PARP-1) and ROS/RNS generation. However, AEA-induced cell apoptosis in primary CTs was TRPV1-dependent, since its antagonists were able to reverse AEA effects on cell viability, caspase 3/7 activity, loss of $\Delta\psi m$ and ROS/RNS production. Moreover, the best characterized TRPV1 agonist, CPS, induced similar effects to those observed after AEA treatment. Supporting these data, TRPV1 activation by CPS has been associated with programmed cell death, in other cell types. It was reported that CPS induces apoptotic-related events such as caspase activation, loss of $\Delta\psi m$, cytochrome *c* release, MAPK phosphorylation or ROS generation [337, 417-421]. In addition, TRPV1 mediates AEA-induced apoptosis in other cell types [153, 336, 337], suggesting a biological role for TRPV1 in cell apoptosis. In fact, and on the contrary of the exogenous CPS, AEA is an endogenous lipid ubiquitously present in several tissues and organs, including the human placenta [395], pointing to a role for the endovanilloid signalling in cytotrophoblast cells turnover. In this way, it is suggested that, besides the ECS, the TRPV1 may intervene in the regulation of CTs apoptosis and integrate the network of molecules that tightly control

this process to allow a successful placentation. It was also observed that both CPS and AEA increase intracellular Ca^{2+} levels, by a TRPV1-dependent way. TRPV1 is a non-selective cation channel but it has preference for calcium ions, suggesting a role for this receptor in regulation of calcium homeostasis. Ca^{2+} ions are important mediators in cell proliferation, differentiation and apoptosis of various cell types, including cytotrophoblasts [422]. Furthermore, the relation between the CPS-induced increase in intracellular Ca^{2+} levels and apoptosis was already described [417, 421]. This information further supports a role for TRPV1 in cytotrophoblast cell apoptosis.

As referred above, eCBs induced an increase in ROS/RNS production in cytotrophoblasts involving the activation of CB and TRPV1 receptors. Although gestation is a natural state of oxidative and nitrative stress, threats to pro-oxidant/antioxidant equilibrium due to increased ROS/RNS or diminished antioxidant defences may negatively impact the biological function of proteins and lipids [423, 424]. Furthermore, alterations in the redox status of placenta have been associated with gestational complications [425-428]. In this way, it is plausible that anomalies on endocannabinoid/endovanilloid signalling may interfere with the balanced placental redox status and be implicated in a poor pregnancy outcome.

In human CTs, AEA induced apoptosis through TRPV1 activation instead of CB receptors activation, as occurred in the case of the cytotrophoblastic BeWo cells. These different mechanisms triggered by AEA may be explained by two reasons. Firstly, BeWo cells are a choriocarcinoma cell line and, though they have been widely used and accepted as a cytotrophoblast cell model, they keep tumour cells properties. Secondly, there is still no consensus if this cell line is representative of cytotrophoblast of first or third trimester of pregnancy, which have distinctive properties, e. g. different hCG secretion levels, proliferative and invasive capacity [429, 430]. Since we assessed AEA effects on CTs from term placenta, it is possible that the different properties of BeWo cells may influence the receptors activated by AEA.

In summary, the results of this study suggest that both eCBs may be novel interveners in the regulation of cytotrophoblast apoptosis, which enlighten a role for these molecules during placentation. In addition, deregulations in endocannabinoid/endovanniloid signalling may have a negative impact in placental development, since altered apoptotic rates and expression of apoptotic proteins have been associated with gestational diseases, such as preeclampsia, HELLP syndrome, IUGR and hyperglycaemia [79, 80, 83, 84, 431-433].

Besides proliferation and apoptosis, the formation of syncytiotrophoblasts (syncytialization) is another critical event for placentation. The syncytialization consists in fusion and biochemical differentiation of the mononuclear CTs. The syncytiotrophoblast is in direct contact with the maternal blood, constituting the barrier between the mother and foetus, which is responsible for gas exchange, nutrient supply and production of hormones and other proteins that are crucial for the gestational success [6]. ST is the main trophoblast cell type that synthesises proteins such as hCG, leptin or ecto-pALP, which are considered biochemical markers of syncytialization. In this work, it was verified that 2-AG impaired the biochemical differentiation of primary human CTs into STs. Indeed, 2-AG decreased the ecto-pALP activity, hCG secretion and the transcription of *LEP,* the gene encoding for leptin. Moreover, these effects were reversed by both CB1 and CB2 antagonists, except for pALP activity, which was only reversed by CB2 blockade. In addition, CPS also impaired this process, as shown by the decrease in ecto-pALP activity and hCG secretion, effects reversed by TRPV1 antagonists. On the contrary to 2-AG and CPS, AEA did not affect hCG secretion, though it decreased pALP activity independently of TRPV1 activation. The different action of AEA in syncytialization may be explained by its agonist activity on other receptors besides TRPV1 and CB receptors, for example the PPAR-γ [163], which is also expressed in human CTs [43]. This AEA promiscuity may implicate the triggering of different signalling pathways, which may be mutually neutralized, masking AEA individual effect on each receptor. Further studies are required to fully understand the AEA role in syncytialization.

hCG and leptin have well recognized pleotropic effects throughout pregnancy and are essential for a proper placental development. Since 2-AG impairs hCG secretion and *LEP* transcription by a CB receptor-dependent manner, it is suggested that, besides interfering with CTs biochemical differentiation into STs, a deregulated endocannabinoid signalling may also impair the processes regulated by these two hormones. Moreover, CPS affects hCG secretion through TRPV1 activation, indicating that TRPV1 signalling influences the secretion of this hormone. Although no linkage between hCG and eCBs has been reported so far, it is known that leptin activates FAAH promoter in human T lymphocytes [365] and leptin knock-out mice have higher uterine levels of AEA and 2-AG [368]. In this work, it was described that 2-AG interferes with *LEP* transcription, suggesting the existence of a mutual regulation between leptin and ECS. Since the ecto-enzyme pALP expression is mainly restricted to the ST, it is widely used as a biochemical marker of syncytialization. It has been associated with the transport of maternal IgG to the foetus and a decrease on pALP activity was noticed in cytotrophoblasts from IUGR placentas

[434, 435]. 2-AG and CPS-induced decrease of its activity supports the effects of these molecules on CT biochemical differentiation.

The participation of CB receptors and TRPV1 in cell differentiation has already been described. Indeed, CB1 activation impairs myoblasts differentiation [176] and stimulates neural progenitor cells [436] and mice trophoblast stem cells differentiation [407]. In addition, TRPV1 inhibits the differentiation of immature dendritic cells [215] and, on the other hand, its activation is important for osteoblasts and osteoclasts differentiation [216] and for myofibroblasts formation from corneal fibroblasts [217]. This information further supports that CB and TRPV1 receptor-mediated signalling may also regulate this important cellular event in human CTs.

The morphological CT differentiation consists in the fusion of the mononucleated CTs to form the syncytial layer. This process is enhanced by the fusogenic proteins syncytin-1 and -2, whose transcription is regulated by the transcription factor GCM-1 [13-16]. In this study, it was demonstrated that 2-AG diminished the number of nuclei in syncytium and the transcription of genes encoding GCM-1 and syncytin-2, through a CB receptor-dependent mechanism.

The cAMP/PKA signalling pathway is considered one of the most relevant pathways that participate in syncytialization, by regulating the synthesis of several proteins. In fact, this pathway intervenes in hCG, leptin and pALP synthesis [50, 71, 96, 104, 437] and cell fusion [51, 72]. It was verified that 2-AG decreased cAMP levels, by a CB receptor-dependent mechanism. Moreover, this effect was reversed by pertussis toxin, an inhibitor of $G_{i/0}$ protein subunits, suggesting an action on $G_{i/0}$ protein-coupled CB receptors. When activated, these receptors decrease AC activity, diminishing cAMP levels, leading to an inhibition of PKA phosphorylation [283]. Supporting these data, it was demonstrated that 2-AG also decreased p-PKA, effect that was reversed by CB1 and CB2 antagonists. Hereupon, the negative impact that 2-AG has in CT differentiation may result from the decreased levels of cAMP and consequently of PKA phosphorylation, after the $G_{i/0}$ protein-coupled CB receptors activation.

The syncytialization is one of the most important events during placental development and anomalies with this event have been related to gestational complications like preeclampsia, IUGR or miscarriages [438-441]. The results of this work suggest that ECS and TRPV1 are new modulators of this process, interfering with the secretion of important pregnancy-related hormones. Thus, threats to ECS or TRPV1 homeostasis may be involved in the patophysiological mechanisms of these diseases, since 2-AG affects not only the biochemical but also the morphological differentiation of CTs into STs.

In conclusion, in this work, it was demonstrated for the first time that the major enzymes responsible for the synthesis and hydrolysis of 2-AG, DAGL-α and MAGL respectively, are expressed in human CTs and STs, allowing an *in situ* regulation of 2-AG placental levels. In addition, the cation channel TRPV1 was also identified in these cells. The major eCBs AEA and 2-AG induced apoptosis and oxidative/nitrative stress in the cytotrophoblast cell model BeWo cells, through the activation of CB receptors. In human CTs, AEA also induced apoptosis through TRPV1 activation. Furthermore, it was reported that 2-AG affects the spontaneous *in vitro* biochemical and morphological differentiation of CTs into STs, by a CB receptor-dependent way that may result from decreased cAMP levels and PKA phosphorylation. Moreover, contrary to AEA, the TRPV1 agonist CPS was also able to impair the biochemical differentiation. In this way, 2-AG seems to be the most relevant eCB in the modulation of syncytialization process.

Together, these results shed light on a role for the endocannabinoid system and TRPV1 in placental development, suggesting that endocannabinoids and endovanniloids are novel interveners in the network of hormones, proteins and other mediators that regulate cytotrophoblast cell proliferation, differentiation and apoptosis. They also indicate that unbalanced endocannabinoid and endovanniloid signalling may have negative consequences in cytotrophoblast cell turnover and, concomitantly, compromise normal placental development. However, there is much more to unveil about the ECS in the human placenta. Future studies may rely on the effects of eCBs on the endocrine function of ST and the invasive properties of EVTs, two topics where the knowledge about role of these lipid molecules is still inexistent.

PART IV
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